Towards identifying markers for post-harvest physiological deterioration in cassava (Manihot esculenta Crantz)

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"TOWARDS IDENTIFYING MARKERS FOR POST-HARVEST PHYSIOLOGICAL DETERIORATION IN CASSAVA (*Manihot esculenta* Crantz)"

submitted by

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is one of the most important staple food crops in tropical countries where it is grown by small farmers largely for their own or local consumption. Cassava suffers a rapid post-harvest physiological deterioration (PPD), starting 24-48 hours after harvest, rendering the roots unpalatable and unmarketable. This is a major constraint of cassava as a crop. PPD is characterised by vascular streaking, a blue-black discoloration that appears as a net of streaks in the vascular parenchyma, spreading to the storage parenchyma and eventually causing a diffuse brown discoloration accompanied by dry lessons. PPD has been associated with mechanical wounding of the root produced during harvesting and transportation. PPD, the product of abiotic stress, has shown many parallels with wound responses in other plant systems. Consequently, a biochemical approach was followed to study the occurrence of secondary metabolites produced by the activation of the phenylpropanoid pathway that may play a determinant role during PPD process. As well, the activity of some enzymes related to wounding responses was evaluated. The study of these biochemical traits was based on the evaluation of cassava cultivars with different reactions to PPD. The identification of key biochemical traits will generate the context and tools necessary for the improvement of cassava germplasm with respect to PPD by means of breeding and genetic modification.
The identification of these "PPD markers" will help to the development of screening methods for use in germplasm evaluation of breeding programs.

Identification of secondary metabolites resulted in the detection of four hydroxycoumarins (scopoletin, scopolin, esculetin and esculin) and three flavan-3-ols ((+)-catechin, (+)-catechin gallate and (+)-gallocatechin). Scopoletin and scopoletin showed the most substantial increases in concentration, peaking two to three days after harvesting. The flavan-3-ols, which showed antioxidant properties, were not related to the early stages of PPD because they started to accumulate four to six days after harvesting, they may be related to microbial deterioration. The biological activity of the phenolic compounds produced during PPD was assayed, identifying scopoletin as an antimicrobial. HPLC and TLC patterns of the secondary metabolites produced during PPD, showed that one peak with non polar properties was related to the onset of PPD. Its chemical structure stills need

to be elucidated. This possible PPD-marker was quantified by means of scopoletin, giving a good positive correlation with the progress of PPD and showing differences between the cultivars with contrasting responses to PPD.

The activity of the oxidative enzymes peroxidase and polyphenol oxidase showed to be related with the onset and progress of PPD. The other enzymes tested, phenylalanine ammonia liase, catalase, β-1,3-glucanase and chitinase did not show a clear tendency during the post harvest time course or their activity started to increase four day after harvesting. Isoelectro focusing electrophoresis identified six peroxidase isoforms, one of which occurred two days after harvesting and increasing in intensity as the progress of PPD only in the high PPD susceptible cultivar. The enzyme activity localisation assays, showed that at the start of post harvest peroxidase is present in the xylem parenchyma and cortex, and then spread all over the storage parenchyma as the progress of PPD.

Knowing the positive correlation of scopoletin and peroxidase with PPD, scopoletin was assayed as phenolic substrate for cassava peroxidases. The reaction resulted in the production of an insoluble dark coloured precipitate, which may be the cause of the discolouration of the vascular parenchyma during post harvest storage. Isoelectro focusing electrophoresis indicated five scopoletin-peroxidase isoforms, three of them increasing in intensity parallel to the progress of PPD. Localisation assays by tissue printing indicated that scopoletin-peroxidase activity is initially concentrated in the vascular parenchyma, following a rapid spreading throughout the root parenchyma as the progress of PPD damage.

Thirty five genotypes of the F1 population (144 individuals) which had been used for the construction of the cassava genomic map were evaluated for the secondary metabolites accumulation and enzyme activities related to PPD. By means of single point analysis between the biochemical traits and molecular markers used in the map construction, three zones of interest for PPD were localised in the female map. Linkage groups D, F and I showed molecular markers significantly correlated with peroxidase, scopoletin peroxidase,

scopoletin and the "PPD-marker". Some of these molecular markers were determined to be associated with quantitative trait loci (QTL) for PPD.

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AA	4-aminoantipyrine
Abs	Absorbance (optical density)
ACMV	African Cassava Mosaic Virus
AUTC	area under the curve
BSA	bovine serum albumine
ButOH	butanolic alcohol
CAROT	carotenoid
CAT	catalase
CATE	(+)-catechin
CATE-G	(+)-catechin gallate
CBB	Cassava Bacterial Blight
CIAT	Centro Internacional de Agricultura Tropical
Da	dalton
dH_2O	destiled water
DHBS	3,5-dichoro-2-hydroxy-benzenesulfonic acid
DM	dry matter
DPPH	1,1-diphenyl-2-picryl-hydrazyl radical
DTT	dithiothreitol
DW	dry weight
ECZ	edapho-climatic zones
ELIN	esculin
ETIN	esculetin
EtOH	ethanolic alcohol
FW	fresh weight
GAE	gallic acid equivalents
G-CATE	(+)-gallocatechin
h	hour
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRGP	hydroxyproline rich glycoprotein
HRP	horseradish peroxidase
IITA	International Institute for Tropical Agriculture
ln	natural logarithm
log	decimal logarithm
LPE	liquid phase extraction
mA	milliAmpere
MilliQ-H_2O	deionised water
min	minute
Na-EDTA	Ethylenediamino tetra-acetic acid, disodium salt
PAL	Phenylalanine ammonia lyase
PEG	polyethylene glycol
PHEN	soluble phenolic content
pI	isoelectric point
POX	peroxidase

PPD	post harvest physiological deterioration
PPD-M	post harvest physiological deterioration marker
PPO	polyphenol oxidase
Prot	Protein
PVPP	polyvinyl polypyrrolidone
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
REGWQ	Ryan-Einot-Gabriel-Welsch Multiple Range Test
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
SCP	scopoletin
SCPOX	scopoletin-peroxidase
SCP-POX	scopoletin-peroxidase
SLIN	scopolin
SPE	solid phase extraction
TAE	tannic acid equivalents
TLC	thin layer chromatography
UV	ultra violet light
V	volt
W	watt

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

1.1 THE CASSAVA PLANT

1.1.1 Taxonomy

Cassava (*Manihot esculenta* Crantz), also known as yuca, mandioca, tapioca, manioc, manioca, aipim, sagu, mhogo and omowgo belongs to the Euphorbiaceae family. This family is characterised by the development of laticiferous vessels, composed of secretory cells called galactocytes from which latex is synthesised. The Euphorbiaceae includes crops with different economic significance as oil producers (*Ricinus communis,* castor bean), latex producers (*Hevea brazilensis*, rubber), edible roots (*Manihot* spp.), weeds (*Euphorbia* spp.), ornamentals and medicinals (Domínguez 1983). Within the systematic hierarchy, the taxonomical classification of cassava is presented in table 1.1.

Class	Dicotyledoneae
Subclass	Archichlamydae
Order	Euphorbiales
Family	Euphorbiaceae
Tribe	Manihoteae
Genus	Manihot
Species	*Manihot esculenta* Crantz

Table 1.1 Taxonomic classification of cassava

The genus Manihot comprises two sections Arboreae and Fructicosease, represented by trees and shrubs respectively, adapted to arid savannas regions (Jennings 1995). Within this genus 98 species have been described, but only *M. esculenta* is commercially cultivated and *M. glassiovii* was cultivated as a source of rubber. Synonyms of cassava are *Manihot utilissima* Pohl, *Manihot aipi* and *Manihot edulis*.

1.1.2 Origin and dispersal

The archaeological evidence and the discovery of pre-Columbian tools used to transform manioc (Fig 1.1) found in Colombia and Venezuela showed that cassava was cultivated 3000 to 7000 years ago (Reichel-Dolmatoff 1957). There is also evidence of presence of starch grains, identical in size and morphology from actual varieties of *M. esculenta*, on the surfaces of stone tools from archeological sites in Panama, dating up

to 8000 radiocarbon years (Piperno and Holst 1998; Piperno et al. 2000). However, the exact region where cassava originated has been difficult to prove. Studies carried out by Renvoize in 1973 showed that cassava could have multiple origins. Spath (1973) proposed 4 zones of origin for cassava: 1) Guatemala and Mexico, 2) coast savannas of north-west America, 3) East of Bolivia and north-west of Argentina and 4) East of Brazil.

Figure 1.1 Fragment of a pre-Columbian cassava grinder found in Colombia

Based on a field study from the south of the United States to Argentina, Rogers and Appan (1973) described 98 species within the genus *Manihot.* These species are distributed along the humid lowlands zones of tropical America. Nassar (1978) proposed four centres of diversity for the wild species: central regions of Brazil, (south of Goias department and west of Minas Gerais department) with 38 species, west of Mexico (19 species), and two additional centres of minor importance, in north-east of Brazil and the west part of Mato Grosso and the other, east of Bolivia. Some authors think that cassava was domesticated after the hybridisation of two closely related wild species (Allem 1994; Rogers and Appan 1973). Based on a study of the variability of chloroplast, ribosomal and nuclear DNA of wild and cultivated species, Fregene et al (1994), proposed that cultivated cassava resulted from the domestication of a wild species, *Manihot esculenta* subsp. *flabellifolia.* Roa et al (1997) confirmed the origin of domestic cassava from the subspecies *flabellifolia* and *peruviana.* Olsen and Schaal (1999) using a phylogeographical analysis, based on a single-copy nuclear gene (glyceraldehyde 3-phosphate dehydrogenase, G3*pdh*), reconfirmed the domestication of cassava derived from the subspecies *flabellifolia* along the southern border of the Amazon basin. Varieties called bitter with a high content of cyanogenic glycosides and

varieties called sweet with low contents of cyanogenic glycosides in the roots are distributed in different regions suggesting that they were domesticated at different moments.

The Portuguese introduced cassava to Africa in the sixteenth century from Brazil (Jones 1959). Today, cassava is largely cultivated in Africa. It is cultivated especially in humid regions, in the west coast, west of Zaire, east coast of Tanzania, Mozambique and in the countries of the great lakes. In Asia, cassava was introduced in the eighteenth century by the Spanish from Mexico. Cassava spread very rapidly from the Philippines to all of Asia and the Indian Ocean (Burkill 1966).

Nowadays, cassava is grown in the hotter lowland tropics and is never grown as a crop further from the Equator than 30 N or 30 S, from sea level to an altitude of 2300 meters (Cock 1985). Cassava shows adaptation to a broad range of conditions, explaining its worldwide distribution. Cassava does best when the rainfall is 100-150 cm per year and well distributed.

1.1.3 Botanical description

Cultivated cassava, *Manihot esculenta* Crantz, is a perennial woody shrub, which can reach 3 to 5 m height (Fig 1.2). During the first months after planting, fibrous roots begin to develop, thicken and store large quantities of starch in the parenchyma.

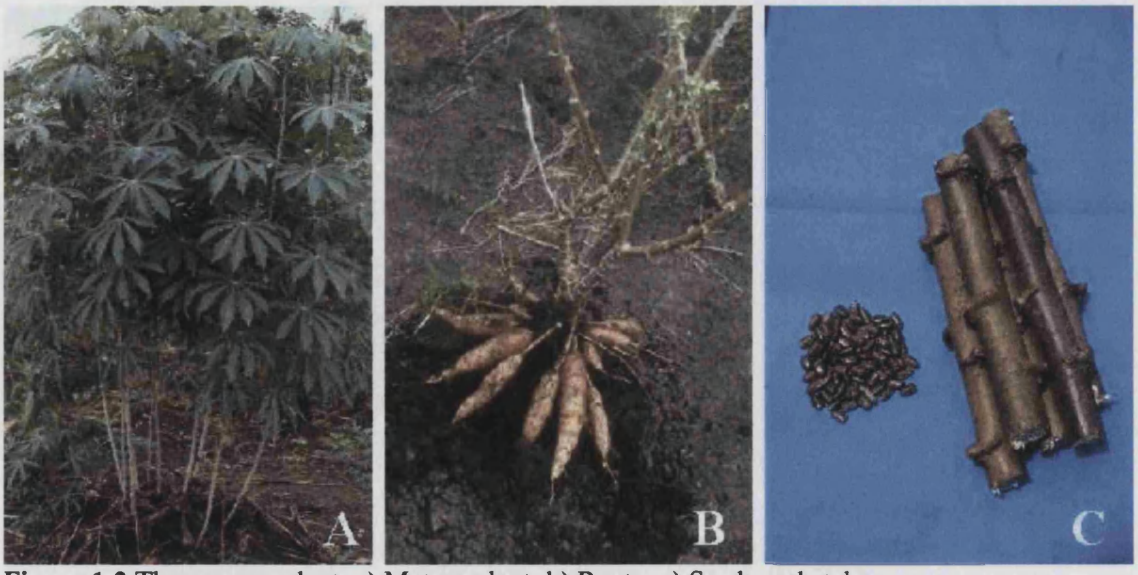

Figure 1.2 The cassava plant. a) Mature plant. b) Roots. c) Seeds and stakes.

Root number and size are very variable among plants. Dry matter of the roots is 90 % carbohydrate, principally starch. Stems are woody and brittle. The coloration of the stem may be whitish, brown or dark-brown depending on the variety. Stem branching during growth occurs at different heights, and is influenced by genotype and environmental conditions. There are basically two types of branching patterns: forked and lateral branching. Leaves constitute a simple lamina with a smooth margin, but are deeply palmate or lobed (3 to 9 lobes). They are held on a nearly horizontal plane, attached to slender petioles at the base of the lamina (Domínguez 1983)

The fruit is a dehiscent, trilocular capsule that is ovoid or globular in shape. It is from 1 to 1.5 cm in diameter and has six straight prominent longitudinal ridges or aristae. Cassava seed is ovoid-ellipsoidal in shape and is approximately 10 mm thick (Domínguez 1983). Cassava is normally propagated vegetatively by lignified stem cuttings called stakes, (15-25 cm long); however, propagation by seeds occurs under natural conditions as well as in plant breeding programs (Cock 1985).

1.1.4 The root system

The cassava root system has a low density of roots but deep penetration, characteristics that provides the crop with the ability to resist prolonged dry periods. Water and nutrients are absorbed by fibrous roots; then few of these roots, generally no more than ten, develop into tuberous roots. The tuberous and fibrous roots are morphologically and anatomically identical. The difference is the change of growth direction, from longitudinal to radical when starch accumulation initiates. The root system of a mature cassava plant consist of fibrous roots, tuberous roots that end in a fibrous root, and a peduncule which connects the roots to the stem from the neck of the tuberous root (Domínguez 1983). The roots differentiate within ten days after the stakes are planted. Storage roots develop by enlargement by the cambium and the formation of secondary xylem followed by the appearance of an outer periderm. About 26 days after planting starch grains are deposited in the secondary xylem parenchyma. Bulking starts from the outer layer to the centre of the storage parenchyma (Wheatley 1982). The roots can be harvested between ten to 12 months after planting. In a cross section of cassava roots, four different regions can be distinguished: the periderm, the cortex, the flesh, and the central vascular strands (Fig 1.3). The periderm, which may vary in colour, thickness and roughness, is formed from few layers of mainly dead cells that effectively seal off the surface of the root. The cortex consists of the tissue (1-4 layers of cells) located immediately below the periderm. It is usually white and, together with the outer

periderm, forms the peel of the root. The flesh is the main storage region of the plant where starch grains are deposited. It constitutes the central portion of the root and consists largely of storage parenchyma cells. However, a few xylem elements and lactifers can be found in the starchy flesh. Finally, the central vascular strands consist of xylem bundles and fibres (Conceicao 1981).

The bulk of a mature cassava root consists mainly of water and carbohydrates. Water content in a fresh root varies from 60 to 70 % depending on the variety, age at harvest, the soil condition, and rainfall. The remainder of the root (dry matter) is mainly composed of starches, sugars and fibres. The nutritional composition of 1 Kg of cassava has been reported as follows: 1460 cal food energy, 625 g water, 347 g carbohydrate, 12 g protein, 3 g fat, 330 mg calcium, 7 mg iron, 0.6 mg thiamine, 0.3 mg riboflavin, 6 mg niacin and 360 mg vitamin C (Cock 1985).

Ketiku and Oyenuga (1972) investigated the compositional changes occurring in cassava root carbohydrates during growth. Using an early maturing variety known as "Oloronto", these authors found that starch accumulation reached its maximum (81 % on a dry weight basis) eight months after planting. A decrease to 78 % occurred when the roots were harvested one month later. This decrease was accompanied by an increase in total sugars from 3.5 % to 5.7 %. Sucrose accounted for the highest proportion of total sugar (77 %), followed by fructose (10 %), glucose (7 %) and maltose (2 %).

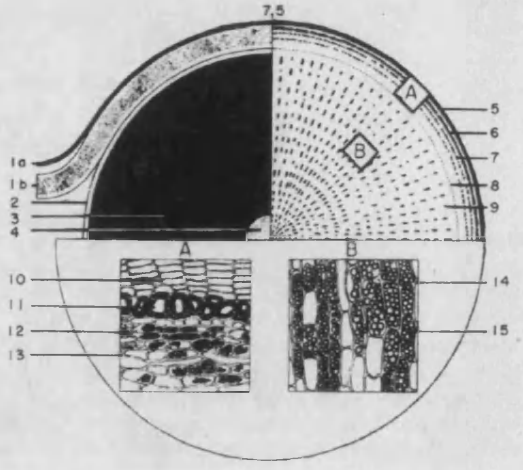

Figure 1.3 Cross section of cassava root (Grace 1977).
Left upper quadrant, after staining with iodine: 1.Peel (periderm), 1a.Outer cork layer, 1b.Inner layer, 2.Cambium, 3.Centre, 4.Pith and primary xylem
Right upper quadrant, showing structural elements of the roots: 5.Cork, 6.Sclerenchymatous fibres, 7.Latex vessels, 8.Cambium, 9.Xylem vessels
Inset A-Enlarged cross section of peel: 10. Cork tissue, 11.Sclerenchymatous fibres, 12. Starch, 13.Parenchyma cell
Inset B-Enlarged cross section of centre:14.Cell wall, 15.Starch

1.2 ECONOMIC IMPORTANCE AND AGRICULTURAL USES

Cassava is a staple food for over 500 million people living throughout the tropics and as a human calorie source cassava has been ranked 4th after rice, sugarcane and maize (FAO 1998). Because much of the cassava grown is produced by small farmers in marginal agricultural areas, it is difficult to obtain exact data on production (Cock 1985). Seventeen million hectares of cassava are grown in the world, with 10 million hectares in Africa, 2.5 million hectares in South America and 3.5 million hectares in Asia (Table 1.2). Cassava production in the world reaches 170 million tonnes/year; 50 % is produced in Africa, 30 % in Asia and approximately 20 % in South America.

Country	Area (Ha)	Yield (Hg/Ha)	Production (Mt)
Africa	10,27	91,330	93,865,848
Angola	523,1	59,821	3,129,734
Congo, Dem Republic of	1,096	145,499	15,959,000
Mozambique	925,9	57,911	5,361,974
Nigeria	3,135	107,987	33,854,000
Tanzania, United Rep of	848,1	67,890	5,757,968
Uganda	382,0	130,000	4,966,000
Zambia	165,0	61,818	1,020,000
Asia	3,535	136,623	48,308,646
China	235,0	159,565	3,750,900
India	250,0	232,000	5,800,000
Indonesia	1,360	116,176	15,800,000
Malaysia	38,00	97,368	370,000
Philippines	210,0	80,952	1,700,000
Thailand	1,150	158,983	18,283,000
Viet Nam	234,9	86,684	2,036,200
Latin America and the Caribbean	2,637	126,132	33,267,569
Brazil	1,753	139,616	24,481,356
Colombia	212,0	84,434	1,790,000
Costa Rica	9,400	129,787	122,000
Cuba	74,00	28,378	210,000
Dominican Republic	19,13	65,354	125,023
Haiti	74,41	44,613	332,000
Paraguay	240,0	145,833	3,500,000
Peru	80,00	110,700	885,600

Table 1.2 Cassava world production in 2001. Source: FAOSTAT, Agriculture data.

The economic importance of cassava is dependent on several factors, 1) cassava produces a higher amount of food calories per hectare than do most other tropical crops under a low input of work and money; 2) even in modern cassava production, the crop requires very little care beyond the stage of canopy closure; 3) the planting material is a

stem cutting, a non-edible part of the plant; 4) cassava is well adapted to traditional mixed cropping which is a security for total crop failure; 5) cassava is very tolerant to drought, a common characteristic found in the tropics; 6) cassava root can be left in the ground for long periods providing a food reserve against famine; 7) cassava has some characteristics that make it a very easy crop for breeding: plant flowers regularly and in some instances continuously, male and female flowers are separated in time (protogyny) and space (monoecism) and the seed set is regular and the seeds germinate very easily (Onwueme 1978; Wenham 1995).

The cassava crop is principally grown for its roots, however in certain areas the leaves are also harvested (e.g.: The Amazon River basin, West Africa, Congo (Zaire) and Tanzania). The leaves can be eaten as fresh vegetable, ground fresh and frozen or dried and ground. Cassava leaves may have high content of cyanogenic glycosides, but are more nutritionally balanced than roots and can help to prevent some deficiency diseases. Due to their higher proportions of proteins, minerals and vitamins, cassava leaves are used to complement the diet in certain regions. The hydrocyanic acid (HCN), released upon damage in the plant, can be lowered to non toxic levels by evaporation during cooking or removing the liquid after pressing ground fresh leaves (FAO and IFAD 2001).

The roots are utilized in many different manners and different quality characteristics are needed for the different end uses (Table 1.3).

	Cassava	Potato	Sweetpotato	Yam	Aroids
Dry matter (%FW)	30-40	20	19-35	21-40	22-27
Starch (%FW)	27-36	13-16	18-28	18-25	19-21
Total sugars (%FW)	0.5-2.5	0-2.0	1.5-5.0	0.5-1.0	2.0
Protein (%FW)	0.5-2.0	2.0	1.0-2.5	2.5	1.5-3.0
Fibre (%FW)	1.0	0.5	1.0	0.6	0.5-3.0
Lipids (%FW)	0.5	0.1	0.5-6.5	0.2	0-1.5
Vitamin A (mg/100g FW)	17	Trace	900	117	0.42
Vitamin C (mg/100g FW)	50	31	35	24	9
Ash (%FW)	0.5-1.5	1.0-1.5	1.0	0.5-1.0	0.5-1.5
Energy (kJ/100g)	607	318	490	439	390
Starch extraction rate (%FW)	22-25	8-12	10-15	na	na
Starch grain size (micron)	5-50	15-100	2-42	1-70	1.12
Amylose (% total starch)	15-29	22-25	8-32	10-30	3.45
Max. Viscosity (BU)	700-1100	na	na	100-200	na
Gelatinization temp. (°C)	49-73	63-66	58-85	69-88	68-75
Anti-nutritional factors	Cyanogens	Solanine	Trypsin inhibitors	Alkaloids, tannins	Oxalate crystals

Table 1.3 Raw material characteristics of roots and tubers (na means not available). Source (Scott et al. 2000)

In general the so-called sweet varieties, which have low levels of cyanogenic glycosides and are concentrated in the peel, have a broader range of uses than the bitter varieties, which have high levels of glycosides that are spread all over the tuberous root. These varieties must be processed before being eaten or used as animal feed.

Cassava is used principally as a human food in Africa. In large parts of tropical Africa cassava is the single most important calorie source being the basic staple for more than 200 million Africans. About 70 % of the cassava is processed before consumption in several different manners to produce various meals, flours, fermented pastes, tapioca, chips and starch. The processing brings some solution to two cassava constraints, toxicity and perishability. Any kind of processing reduces the cyanogenic glycosides levels, and the processing into dry products avoids perishability (Cock 1985). In Latin America cassava is important in certain areas as a human food, however it is increasingly being used as a source of starch or as an animal food. Brazil is by far the largest producer of cassava in Latin America and uses cassava in many forms, including fresh cassava, farinha, fermented starch, starch and dried cassava as an animal feed. The use of cassava varies greatly throughout Asia. In Kerala, Southern India, it is important as a dietary staple as fresh cassava, whereas in the neighbouring state of Tamil Nadu it is an important source of starch. Thailand processes virtually all the cassava to produce either starch or dried cassava pellets for export as animal feed. Cassava is also dried and exported and in Sumatra there are several large starch factories. In Vietnam cassava is becoming more important as a crop with much of the production dedicated to small-scale starch production and as a local source of animal feed.

The simplest way to process fresh cassava is cooking. Cassava is peeled after harvesting and then boiled. During boiling the linamarase is inactivated but linamarin is not degraded, hence a long-term ingestion combined with a diet low in protein and iodine may evolve in chronic cyanide toxicity (Cock 1985). The multiple forms of cassava meals are divided into unfermented and fermented meals. Unfermented meals follow a basic process that consist of peeling and washing the fresh cassava roots, grating or grinding, dewatering by pressing, braking the pressed mash, drying and milling (Onabulu et al. 1998). In Africa, cassava is often immersed in pools of water for some days until the roots get soft, then they are peeled and sun dried before grinding. Gaplek (Indonesia) is prepared from dried chunks that are ground; it is used particularly by the poorer segments of the population. Kokonte (Ghana) is prepared

from chunks but they are not ground immediately after drying. Farinha de raspa (Brazil) is grated and pressed cassava that is dried in wood-fired or oil-fired furnaces (Fig. 1.4). Chickwangue is another African product in which cassava is soaked in water until it softens, then is peeled and mashed. After elimination of fibres, the cassava dough is wrapped in cassava leaves. It may be consumed as it is or boiled. Casabe is produced in the Caribbean and north coasts of Colombia and Venezuela. The process method is similar to farinha, but the pressed cassava mash is moulded into a flat cake, which is baked to make a tortilla-like bread (Cock 1985). The preparation of gari (Africa) is very similar to farinha but it includes fermentation. After washing, peeling and grating the mash is transferred into bags and squeezed. The bags are stored for some days, period during which fermentation occurs. Finally the fermented mash is roasted or fried. The mash used in the preparation of gari can be fermented under water and then dried to prepare fufu. Farihna and gari can be processed at home, village and large-scale levels. The baked products where cassava is the basic ingredient are known commercially as tapiocas. In Malaysia these products are known industrially as sago products. Tapiocas are prepared from partly gelatinised cassava starch. They are produced in different forms, round beads (seeds or pearls) or irregular lumps (flakes) (Grace 1977).

Large quantities of cassava roots and waste are used for animal feeding. Dried cassava has been successfully used in Europe as an ingredient for animal feed. The most common form of sun dried cassava used in animal feeding is cassava chips. They are dried irregular slices of roots that differ in size. Cassava chips are extensively produced, marketed and exported in Malaysia, Thailand, Indonesia and some countries of Africa. Other forms of processed cassava for animal feeding are broken roots, pellets and residual pulp. Broken roots are thicker and longer than chips and are principally produced in Africa. Pellets are processed from dried and broken roots by grinding and hardening into a cylindrical shape. Residual pulp is obtained during the processing of cassava flour by the separation of the pulp from the starch. The residual pulp is often used wet in the areas around the factories, but can be sun dried before marketing.

There are small-scale starch industries in tropical countries, which have real socio-economic importance for the local economy. In Colombia and Brazil naturally fermented starch is produced for use in the preparation of traditional breads. Examples of this small-scale industries, that are a traditional activity, are: kupruk in Indonesia, sago in India, pan de bono and pan de yuca in Colombia, biscoicho and pao de queijo in

Brazil and chipa in Paraguay (Best et al. 1992; Wenham 1995). Cassava starch can be used in different ways, directly or as raw material. There are principally three kinds of starch products: 1) Unmodified or native starch; 2) Physical, chemical, and biological modified starches for industrial applications (baby and hospitalised persons foods, non allergenic products and pharmaceuticals, paper and textiles, glues and adhesives, alcohols and acetones; 3) Sweeteners (high fructose syrup, dextrine, monosodium glutamate, etc.) (FAO and IFAD 2001; Grace 1977).

The increasing potential for dried cassava market as animal feed, source of starch and speciality foods has decreased the market for fresh roots. Additionally, urbanization provides bigger market opportunities for dried cassava products rather than fresh cassava (FAO and IFAD 2001).

Figure 1.4 Processing of farinha (Bahia, Brazil). Pictures show peeling of fresh harvested roots, grinding and drying.

1.3 CASSAVA COLLECTIONS, BREEDING PROGRAMMES AND GENETICS

1.3.1 Cassava collections

The largest cassava germplasm collection is that of CIAT with close to six thousand accessions of cassava and wild species of *Manihot*. The CIAT collection has a global coverage but is particularly rich in traditional varieties from Latin America. IITA, Nigeria, has traditional African varieties and a large number of lines from crossing programs in Africa. EMBRAPA, Brazil, has collected many of the local varieties from very diverse habitats and CTCRI, India, and SARI, Uganda, have collections focussed on local materials. These are all substantial collections each with more than one thousand accessions. Considerable local genetic variability is also maintained in the collections maintained in Thailand (Rayong Station) and in Indonesia (Cock 1985).

The costs related to maintenance and exploitation of genetic resources of these collections lead to the establishment of a cassava core collection (Hershey et al. 1994). At CIAT, core collection consists of 630 accessions selected following four criteria: 1) geographic origin (the most important), 2) the diversity of morphological descriptors, 3) diversity of α-β-esterase banding pattern, and 4) *a priori* selection of accessions based on a predetermined criterion of specific interest (CIAT 1987-1991).

The main *in situ* collections are those maintained by the tribal populations in the Amazon basin; modern life styles are endangering these collections (Cock 1985).

1.3.2 Breeding programmes

In general, selection of interesting clones is made among the pool of landraces. Breeding of cassava is recent and has four main goals: increased yield, resistance to most important pests and diseases, adaptation to new cultural conditions (early bulking, tolerance to different soil types) and increased quality of roots (starch content, toxicity) (Cock et al. 1979; Lozano et al. 1980). Two different mechanisms are used to increase genetic variability in cassava: controlled pollination and open pollination in polycross blocks. The former is a less expensive means to produce large quantities of seeds and the resulting progeny can be used to measure parental clones combining ability and direct selection. The latter one, permits more control over recombination of specific characters and is most useful where a clone needs to be improved for specific traits (CIAT 1983).

Given the broad range of conditions under which cassava is grown, and the difficulty to develop genotypes adapted to all the production situations, the cassava breeding programme works on developing gene pools adapted to particular regional needs (CIAT 1987-1991). Cassava growing regions are very diverse in their edapho-climatic characteristics. Results collected during several years of research on the interactions between cassava plant and the environment lead to the identification of seven major edapho-climatic zones (ECZs) (CIAT 1983). ECZs were defined based on the world importance of the ecosystem, climatic conditions, predominant soil type and related constraints, pest and disease problems and use of end products (CIAT 1983). Table 1.4 presents a description of gene pools for defining cassava germplasm development in each ECZ. Cassava breeding program in South America is based on the selection of varieties adapted to each ECZ. Later the gene pool description was modified based on the recognition of the growing importance of semiarid regions for expanding cassava

production and the available genetic variability for enhanced water use efficiency. The other modification is based in the growing concern about the effects of HCN on human health (CIAT 1987-1991). ECZs were initially described in Colombia but a comparison of major ecosystems led to the identification of ECZs in Brazil and Venezuela (CIAT 1992).

ECZ	Description	Principal constraints
1	Sub-humid tropics with long dry season, low moderate annual rainfall, high year round temperature	Drought, mites, thrips, termites, mealybugs, bacteriosis, root rots and viruses.
2	Acid-soil savannas with moderate to long dry season, low relative humidity during dry season	Low soil fertility, drought, bacteriosis, *Cercospora* leaf spot, superelongation, anthracnose, mites, mealybugs and lace bugs
3	Humid tropical lowlands with no pronounced dry season, high rainfall, constant high relative humidity	Low soil fertility, *Cercospora* leaf spot, superelongation and root rots
4	Mid-altitude tropics (800-1500 msl) with moderate dry season and temperature	Mites, thrips, mealybugs, bacteriosis, root rots, mycoplasma, anthracnose and viruses.
5	High-altitude tropics with mean temperatures of approx. 17-20°C	Low temperature, *Phoma* leaf spot, anthracnose and mites
6	Subtropics with cool winters and fluctuating day lengths	Low winter temperature, bacteriosis, superelongation and anthracnose
7	Semiarid areas	Drought

Table 1.4 Edapho-climatic zones (ECZ) for cassava production. General description and principal yield constraints.

The development of cassava germplasm is carried out through adaptive and recurring selection of the material in representative areas of different ECZs. The best clones (named "elite clones ") are selected for their adaptation to the specific conditions of the ECZ and then transferred to breeding programs in other areas presenting identical edapho-climatic and ecological conditions. There are basically four selective criteria for cassava breeding. The principal criterion is root yield. This standard is often used as an indirect marker for adaptability and tolerance to biotic and abiotic factors. The second criterion is the root dry matter content. It is growing in importance due to increasing cassava industrialisation. The third criterion is the culinary quality, in the regions where fresh market is the principal use for cassava roots. In the fourth place there are the plant and root characteristics: planting material availability, type of plant (i.e. ramification, which may affect association capacity with other crops), harvesting facility, root colour, root size, etc. (Iglesias 1994).

1.3.3 Genetics

Cassava plants have a chromosome number 2n=36. Polyploids are not common (Onwueme 1978). The allopolyploid origin of cassava is suggested by its possession of two sets of dissimilar nucleolar organizing regions, on the repetition of chromosome types (Magoon et al. 1969; Umanah and Hartmann 1973), and on the basic chromosome numbers of other genera in the Euphorbiaceae (from 6 to 11) (Perry 1943). Heterozygosity is characteristic of cassava (Kawano et al. 1978). Cassava is a strongly outcrossing monoecious species and suffers from inbreeding depression.

A molecular genetic map was developed at CIAT with different types of markers (RFLP, RAPD, microsatellites and isoenzymes) and constitutes a very helpful tool to elucidate genome organization in cassava and to localise different genes (Fregene et al. 1997). The F1 cross was made between "TMS 30572" (MNGA 2) and "CM2177-2', elite cassava cultivars from Nigeria and Colombia respectively (Fregene et al. 1997). Recently, cassava bacterial blight (CBB) infection was evaluated in the 150 individuals of the F1 population under greenhouse and field conditions (Jorge et al. 2001; Jorge et al. 2000). Eight QTLs (quantitative trait loci) were found to explain 9-20% of the phenotypic variance of cassava response to CBB under greenhouse conditions (Jorge et al. 2001). Under field conditions, eight QTLs were found to be involved in resistance to CBB (Jorge et al. 2001).

1.4 CASSAVA CONSTRAINTS

1.4.1 Pests and diseases

African cassava mosaic virus is probably the most important cassava viral disease. Yield losses vary and can reach 45%. Virus is transmitted by a whitefly (*Bemisia tabaci*) (Puonti-Kaerlas 1998). In humid tropics, disease incidence is highly correlated to the populations of the vector agent (Fargette et al. 1993). The use of resistant cultivars is the most appropriate and realistic measure of control. Two other major viral diseases are cassava common mosaic virus (CsCMV) and East African cassava mosaic virus (EACMV).

Three bacteria attack the aerial parts of a cassava plant, and two are species of *Xanthomonas*, *Xanthomonas axonopodis* pv. *manihotis* and *X. axonopodis* pv. *Cassavae*. The former is a widespread disease, found in all regions where cassava is cultivated and the latter is only present in Africa. *X. a.* pv. *manihotis* (*Xam*) is the

causal agent of cassava bacterial blight (CBB) and *X. c.* pv. *cassavae* causes angular leaf spot. CBB shows a wide variety of symptoms, angular leaf spots, blight, gum exudation, stem cankers, shoot wilt, vascular necrosis and die-back (Lozano and Sequeira 1974). In South America, the bacterium *Erwinia carotovora* var. *carotovora* cause bacterial stem rot and may produce severe yield losses (Lozano and Belloti 1978). Besides ACMV and CBB, plant pathogenic fungi cause the most important cassava diseases. Twenty diseases caused by plant pathogenic fungi have been described (Hirose 1986). The most important is root rot, caused by at least three species of *Phytophthora*, *P. drechsleri*, *P. erythoseptica* and *P. cryptogea* (Lozano and Booth 1985). Root destruction can also be caused by: *Diplodia manihotis*, *Armillaria spp.*, *Fusarium spp.* and *Verticillium dahliae.* On leaves, some wilts are also observed and caused by *Colletotrichum gloeosporoides*, leaf spots caused by *Cercosporia henninsii* and *C. viscosae* and super-elongation symptoms caused by *Sphaceloma manihoticola.*
Frog skin root disease, causal organism unknown, have been reported to cause 90% of yield reduction in Colombia (CIAT 1974). The disease appears to affect the normal deposition and storage of carbohydrates in the roots thus the affected plants produce fewer and frequently deformed swollen roots.
Insect pests include cassava mealy bug (*Phanacoccus manihotis*), whitefly (*Bemisia tabaci*), green and red spider mites (*Mononychellus tanajoa*, *Tetranychus telarius*) and root knot nematodes (*Meloidogyne incognita*).

1.4.2 Cyanogenesis

Cassava is a cyanogenic crop. All tissues, except the seeds, contain the cyanogenic glycosides, linamarin and lotaustralin. Cyanogens can be present as well as cyanohydrin and as free cyanide. Leaves have the highest levels (5 g linamarin/kg fresh weight), whereas roots have 20-fold lower linamarin levels (White et al. 1998). Cyanogenesis is initiated when the tissues are damaged. Linamarin is hydrolyzed by the ß-glucosidase linamarase forming acetone cyanohydrin, which can be broken down into acetone and toxic hydrogen cyanide enzymatically by hydroxynitrile lyase (Elias et al. 1997), or spontaneously at pH 5 or temperatures >35°C (White et al. 1998). All cassava cultivars contain cyanogenic glycosides that are eliminated by removal of the outer layer, soaking, boiling or drying before consumption. Health disorders are associated with long term exposure of cassava containing residues of cyanogenic compounds, such

as goitre, hyperthyroidism, tropical ataxic neuropathy and konzo (Rickard and Poulter 1992).

1.4.3 Post-harvest deterioration

One of the main constraints of cassava production is the rapid post-harvest deterioration starting within 48 hours after harvest, which renders the root unpalatable and unmarketable for consumption or industrial uses. Urbanization is strengthening post-harvest deterioration as the major constraint for the development of farmers and processors, because of the large distances between the field and the market. Post-harvest losses in cassava production in Latin America and the Caribbean and Asia reach 10 and 8% respectively, while in Africa they are 29% (FAO and IFAD 2000).

The post-harvest deterioration process in cassava occurs in two stages: primary or physiological post-harvest deterioration (PPD) and secondary or microbial deterioration (Booth 1976; Plumbley and Rickard 1991; Taniguchi et al. 1984b).

1.4.3.1 Primary or post-harvest physiological deterioration (PPD)

PPD is characterised by vascular streaking (Fig. 1.5), which is a blue-black discoloration that appears as a net of streaks in the vascular parenchyma, spreading to the non vascular tissue and eventually causing a diffuse brown discoloration accompanied by dry lesions (CIAT 1973). Prior to the general discoloration of the storage parenchyma an intense UV fluorescence in the storage tissue is observed. The explanation of this phenomenon is the accumulation of secondary metabolites such as coumarins that present blue fluorescence under UV light.

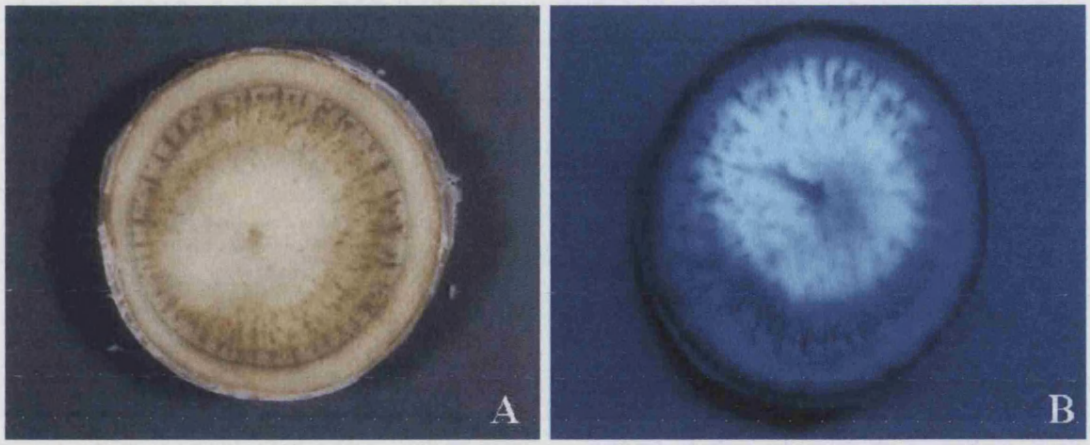

Figure 1.5 Cassava root slices showing symptoms of PPD. A) Vascular streaking and browning of storage parenchyma. B) Blue fluorescence of storage tissue observed under UV light.

1.4.3.2 Secondary or microbial deterioration

Secondary or microbial deterioration starting after 4-5 days of harvesting is associated with a decay caused by invasion of microorganisms through wounds produced during harvesting and handling of the roots. It is considered less important than PPD as it generally occurs after the roots have already become unacceptable as the result of PPD (Booth 1977a).

Microbial deterioration is characterised by rotting, fermentation and softening of the root. Softening starts at the root central core and gradually expands towards the outside. Decay may be accompanied by vascular discoloration, but it has been demonstrated that the vascular streaking produced during PPD is not a response to pathogen attack (Booth 1976; Rickard et al. 1979). Table 1.5 shows microorganisms isolated from damaged cassava roots (Booth 1977a).

The storage methods developed for reducing PDD may also be useful for the prevention of microbial deterioration. For example storage at low temperatures reduces the level of rotting and curing prevents the entry of wound pathogens by the production of periderm (Booth 1977a). A study conducted by Ikediugwu and Ejale (1980) revealed that the removal of potential pathogens from the root surface by surface sterilization also enhances the opportunities of wound healing with the consequent exclusion of most airborne pathogens when roots are stored in polyethylene bags.

Organism	Disease
Bacillus sp.	Minor wet rot, Post-harvest secondary deterioration
Corynebacterium manihot	Root fermentation
Armillaria mellea	Young root necrosis, Minor dry rot
Aspergillus spp.	Post-harvest secondary deterioration
Circinella sp.	Post-harvest decay
Clitocybe tabescens	Root rot
Cylindrocarpon candidum	Post-harvest secondary deterioration
Diplodia manihotis	Root rot
Erwinia sp.	Minor wet rot, Young root necrosis
Fusarium spp.	Minor wet rot
Ganoderma pseudoferrum	Red root rot
Geotricum candida	Root fermentation
Helicobasidium compactum	Minor dry rot
Lasiodiplodia theobromae	Post-harvest secondary deterioration
Mucor sp.	Post-harvest decay
Penicillium spp.	Post-harvest decay
Pheolus manihotis	Root rot
Phytophtora spp.	Young root necrosis, Wet rot
Pythium sp.	Young root necrosis, Minor wet rot
Rhizoctonia sp.	Root rot
Rhizopus spp.	Post-harvest secondary deterioration
Rigidopurus lignosus	White root
Rosellinia spp.	Black rot
Sclerotinia sp.	Young root necrosis
Sclerotium rolfsii	Young root necrosis, Minor dry rot
Sphaceloma manihoticola	Minor root rot
Sphaerostilbe repens	Root rot
Syncephalastrum sp.	Post-harvest decay
Trichoderma sp.	Post-harvest secondary deterioration
Xanthomonas axonopodis	Cassava bacterial blight, Minor dry rot
Unknown	Frog skin disease
Physiological	Post-harvest primary deterioration
Physiological	Hollow heart or core rot
Physiological	Abnormal rot /stem thickening
Physiological	Root greening

Table 1.5 Microorganisms isolated from damaged cassava roots

1.5 POST-HARVEST PHYSIOLOGICAL DETERIORATION (PPD)

1.5.1 Previous studies on PPD

PPD is an endogenous physiological phenomena, as no miroorganisms have been isolated from the tissues showing vascular streaking symptoms (Booth 1976; Noon and Booth 1977). In addition, inoculation of roots with microorganisms isolated from rotted

roots did not induce the symptoms of vascular streaking (Noon and Booth 1977). Mechanical injury during harvesting and handling has been related with the occurrence of vascular streaking (Booth 1976). Marriot et al. (1978) concluded that water stress was a necessary condition for the development of vascular streaking, based on the observation that root portions stored at low relative humidity had higher vascular streaking damage than those subjected to the same physical damage and aeration conditions but held at high relative humidity. Mechanical damage increases water loss and eases O_2 access into the tissue. Thus, three factors have been suggested as the major components in the development of vascular streaking: mechanical injury, oxygen availability and water stress. Additionally, other studies suggest that PPD may exhibit a wound-induced senescence response. "Changes in cassava lipids with ageing could cause structural alterations in cassava membranes, which may allow reactants and enzymes involved in darkening reactions to come into contact, thus leading to parenchyma discoloration" (Lalaguna and Agudo 1989). Treatments such as immersing cassava roots in hot water (53°C during 45 min) inhibit PPD over a storage period of five days, this inhibiting response implies an enzymatic basis for PPD (Averre 1967).

Electron microscopy and cytochemical studies showed that the pigmented materials originated from cells adjacent to the xylem vessels via breaks in the pit membrane. As well, the formation of tyloses was observed in the pit area of pigmented and non-pigmented vessels (Fig. 1.6). These observations led to the conclusion that tylose formation was due to a non-specific wound response and it was not essentially related to the development of vascular streaking (Taniguchi et al. 1984b; Rickard et al. 1979). The pigmented material was found to contain lipid, lignin and carbohydrate and be derived from adjacent parenchyma cells (Rickard et al. 1979; Rickard 1982). A later study carried out by Rickard and Gahan (1983) did not corroborate the presence of lignin in occlusions. The occurrence of lignin-like material is probably produced by condensed tannins with lignin-like properties, formed by the polymerization or condensation of catechins and leucoanthocyanidins. As well, it was observed that the occlusions increase in frequency in wounded roots stored at low humidity. As a result of those observations, Rickard defined vascular streaking as a non-specific wound response that increases when roots are stored at low relative humidity.

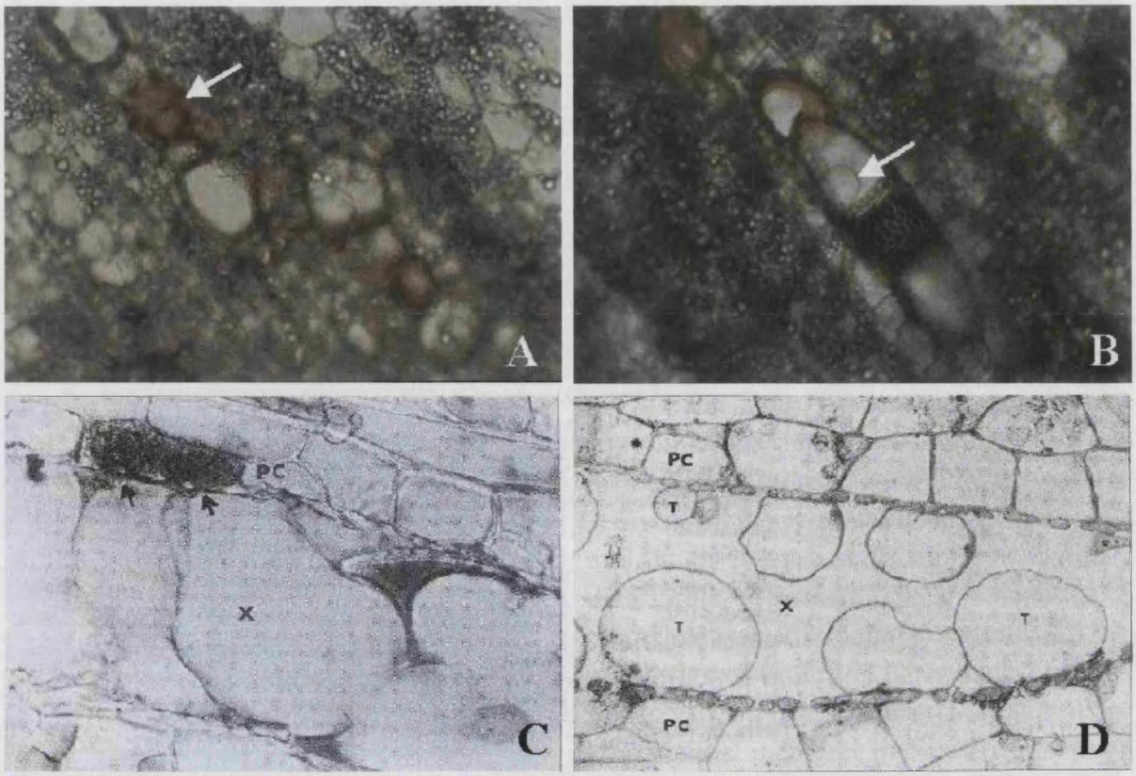

Figure 1.6 Microscopic cross section of cassava root showing symptoms of PPD. A) Occlusion of pigmented material adjacent to xylem vessels. B) Xylem occlusions (tyloses). C) Pigmented material entering xylem vessel (X) from adjacent cell (PC) via pit area (arrow). D) Occluded xylem vessel X containing tyloses (T) originating from adjacent xylem parenchyma cells (PC). Pictures C and D, source: Wenham 1995.

PPD, the product of abiotic stress, shows many parallels to wound responses. During PPD respiration is induced in harvested cassava roots (Hirose 1986). Respiratory rates reached two peaks, the first (related to wound respiration) one day after harvesting, and the second (related to biochemical changes associated to development of PPD) four or five days later. Respiration results in the hydrolysis of starch (Uritani et al. 1984a). The increase of respiration has been positively correlated with the production of ethylene. This phyto-hormone has been found in high levels before vascular streaking and during PPD process (Hirose 1986; Tanaka et al. 1984). A higher production of ethylene was detected in the cortical parenchyma than in the storage parenchyma where vascular streaking occurs (Hirose et al. 1984b). This observation may suggest that ethylene can act as a volatile messenger in signal transduction as in other studied plants. The direct effect of ethylene over PPD was not clear, since the application of exogenous ethylene to cassava roots did not enhance the occurrence and development of vascular streaking (Hirose et al. 1984b). Pre-harvest pruning has been seen to delay PPD, but no clear differences between pruned and unpruned plants with respect to ethylene

production and respiratory rates was observed (Hirose et al. 1984a; Hirose et al. 1984c). Ethylene might affect tissue discoloration by altering respiration and changing peroxidase enzymes (Plumbley et al. 1981).

In most plants tissue damage, produced during harvesting and handling, results in a cascade of wound responses that result in the defence of the wounded tissue (Bowles 1990; Wenham 1995). Plant defence involves pre-existing physical barriers, periderm (Agrios 1988), and the induction of their synthesis to seal the exposed tissue. These barriers are the cell walls, which include the polymers lignin, suberin and callose. Defensive wound responses include the activation of lytic enzymes such as chitinases and ß-1,3 glucanases, protease inhibitor proteins and hydroxyproline-rich glycoproteins (Bowles 1990).

Cell death is another response to biotic and abiotic stress. When the plant controls the initiation and execution of the cell death, the process is named programmed cell death (PCD) (Dangl et al. 2000). This process is known as apoptosis in animal cells. Two types of PCD in plants are senescence, which, as mentioned before, has been associated with PPD in cassava, and the hypersensitive response (HR). Plant senescence is a relatively slow cell death of tissues, comprising the ordered disassembly of cellular components allowing the rescue of nutrients from the senescing tissues for recycling to other plant organs that survive (Dangl et al. 2000). During senescence increased levels of reactive oxygen species (ROS) have been detected. This increase may be due to the enhanced production of the reactive species or a decay of the efficiency of the defence mechanisms that provide protection against oxidative damage (Thompson et al. 1987). The increased production of ROS in senescent tissues maybe a cause of lipid peroxidation and the activity of lipoxygenase (Pauls and Thompson 1984). HR is a localized cell death that blocks the spread of the infection in biotic stresses. The objective of this rapid and localized cell death is to kill the host tissue before the pathogen establishes in the host, so in HR the recovery of nutrients is not very important. In HR the production of H_2O_2 provokes a rapid crosslinking of the cell proteins hampering the invasion of the pathogen (Baron and Zambryski 1995). It has been also observed that H_2O_2 in HR triggers local cell death and acts as a diffusible signal inducing H_2O_2 defence genes (Levine et al. 1994).

The induction of the phenylpropanoid pathway plays a determinant role in PPD because it involves several aspects of the wound response, such as the production of flavonoid pigments, allelochemicals, antioxidants, metal chelators, UV protectants, phytoalexins,

signalling molecules and polymers (lignin and suberin) (Cooper-Driver and Bhattacharya 1998; Dixon et al. 1994; Dixon and Paiva 1995). Phenylalanine ammonia-lyase (PAL) is the key entry enzyme between the shikimate pathway (primary metabolism) and the phenylpropanoid pathway (secondary metabolism) (Solecka 1997). PAL increases in activity during PPD leading to the accumulation of phenolic and polyphenolic compounds in cassava roots (Campos and de Carvalho 1990; Tanaka et al. 1983). The principal phenolic compounds identified in cassava are scopoletin, scopolin, esculetin, esculin, (+)-catechin, gallocatechin, flavanols, proanthocyanidins and tannins (Beeching et al. 1998; Rizk 1987; Sakai et al. 1994; Tanaka et al. 1983; Uritani et al. 1984b; Wheatley 1982). Some of these compounds fluoresce under UV light or are pigmented, so they can be involved in PPD symptoms. The role of scopoletin, a blue coumarin UV fluorescent metabolite, in PPD has been well studied. Scopoletin accumulation starts at the site of damage but spreads to the entire root (Wheatley 1982). Its application to fresh harvested roots seems to accelerate PPD by producing a rapid discoloration, within 12 hours, of the vascular and parenchymal tissues (Wheatley and Schwabe 1985). Other phenylpropanoid compounds were applied to cassava roots but only scopoletin accelerated vascular streaking. Those secondary metabolites were *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, coumarin, umbelliferone, esculetin, arbutin, and catechol. Roots from pruned plants, two to three weeks before harvesting, responded to exogenous scopoletin in the same way as roots from unpruned plants. Cured roots were not susceptible to exogenous scopoletin. These observations lead Wheatley and Schwabe to assume that oxygen absence may induce the loss of a scopoletin precursor or an inactivation of a scopoletin metabolism related enzyme; and pruning may induce the reduction of internal scopoletin accumulation or the absence of a factor involved in prymary oxidation. Scopoletin exhibits antifungal activity against *Corynespora cassiicola* in *Hevea braziliensis* (Obidoa and Obasi 1991) and *Alternaria altenata* in cassava (Taniguchi et al. 1984a). Scopoletin as well exhibits pharmacological effects, is it a potent hypotensive and non-specific spasmolytic agent. These effects are thought to be the underlying factors in a slowly tropical neuropathy endemic among populations subsisting on cassava diets. This neuropathy presents symptoms like optic atrophy, nerve deafness and ataxia. Until now, this pathology had been associated with cyanide toxicity (Obidoa and Obasi 1991). Other enzymes are also induced during cassava PPD, including peroxidases and polyphenol oxidases (Campos and de Carvalho 1990; Kato et al. 1991; Plumbley et al.

1981). These enzymes oxidise phenolic products of the phenylpropanoid pathway resulting in tissue browning. Polyphenol oxidases also play an important role in respiration by transferring electrons from respiratory substances to other hydrogen or electron acceptors (Goodman et al. 1986). Peroxidases and catalases also act in plant defence by removing hydrogen peroxide (Bowles 1990). The oxidation of phenolic compounds can result in the formation of quinones that can be toxic to micro-organisms or can polymerise to produce melanin that can react with amino groups to produce brown precipitates (Beeching et al. 1998).

Secondary metabolites derived from the mevalonic acid pathway have been detected in cassava roots undergoing PPD. These metabolites are diterpenoids, not common stress metabolites, and steroids that can act as plant growth regulators and phytoalexins (Godman et al. 1986). The 22 diterpenic metabolites isolated from cassava, most of them novel, were classified into four families *ent*-beyerane, *ent*-pimarane, *ent*-atisane and *ent*-kaurane (Sakai and Nakagawa 1988).

As seen above, metabolic pathways, enzymes and secondary metabolites are induced in cassava by wounding as in other plants. But the wound repair that finally stops the wound-response signal seems to be ineffective in cassava roots. Consequently, the wound response spreads all over the root instead of concentrating at the wound site (Beeching et al. 1995).

1.5.2 Genetic variability for PPD

Studies on cassava susceptibility to PPD have revealed genetic and environmental effects over the PPD response. Screening of CIAT cassava collection genotypes, during 1973 and 1974, found a small percentage of genotypes that did not deteriorate after two weeks. But the strong positive correlation between PDD and dry matter dissuaded breeders from selecting germplasm material with low PPD. Thus, subsequent research centred in developing post-harvest storage techniques (CIAT 1983).

Montaldo (1973) studied the PPD response of 65 cultivars in two harvesting seasons. The cultivars showed a high variability in the rate of development and severity of vascular streaking, as well he observed in some cultivars different response between the two seasons. He concluded by recommending two varieties as valuable sources of PPD resistance for breeding programs. In order to confirm the Montaldo observations, Booth (1976) studied the PPD reaction of 15 cultivars from CIAT germplasm bank. Roots of all cultivars showed vascular streaking after seven days of storage, but there were

significant differences in the time of PPD beginning and the rate of PPD advance. It was observed, as well, that cultivars differed in perishability independently of the severity of damage. Rojanaridpiched and Kawano, cited by Booth (1977a), tested PPD reaction in 2312 lines of F1 cassava hybrids. 2.12 % presented very low susceptibility to PPD following open storage in the field for 14 days and that out of 232 cultivars examined, three showed high resistance. As well, they saw a normal distribution of the PPD response among the genotypes. These highly resistant genotypes only presented vascular streaking around the injury sites. Fukuda and colleagues (1979) testing post-harvest deterioration over 86 cultivars concluded that this is a genetically controlled character; and that the genetic variability of cassava for PPD is wide enough to select material for breeding programs.

PPD evaluations carried out over 26 cultivars in two different localities, CIAT-Palmira and CIAT-Popayán, did not find significant correlation between both localities. Those results prove that a cultivar can not be classified as susceptible or resistant to PPD without pointing out the locality and environmental conditions in which it was cultivated (van Oirschot et al. 2000). Continuing with the idea of studying PPD reaction in different localities, Iglesias et al (1996) performed PPD evaluations in three localities in Colombia (Palmira, Villavicencio and Media Luna). They observed a significant clone by site interaction, but despite this they considered it possible to select cultivars with constant low PPD reaction across different eco-systems. As a result of this research, the authors intend to cross genotypes with contrasting responses to PPD to construct the basis to study the genetics behind the biochemistry of post-harvest deterioration.

1.5.3 Post-harvest storage treatments

A diverse number of storage techniques have been developed in order to delay the onset of PPD, though some of them are not economical, or not easy viable or inappropriate for large scale marketing.

The simplest method to prevent PPD is to leave the roots in the ground until needed. This method has the huge disadvantage of withholding vast areas of land to the farmer from alternative production (Ingram and Humphries 1972). The roots may increase in size, but they become fibrous and woody (Ravi et al. 1996). In the Amazonian cassava roots are buried in pits or trenches, where the roots can be stored for a few months if the conditions are favourable (Ravi et al. 1996). Soil clamp storage showed that roots can

be stored for periods of one to three months, depending on clamp design and ambient conditions (cool and moist periods). Under hot and dry environmental conditions the roots can not be stored for longer periods than one month (Booth 1975). Studies carried out at CIAT, during 1973, showed that under some circumstances it may be advantageous to remove roots from clamps after two weeks and then store them in boxes than leave the roots for longer periods in the clamps. Packing roots with moist materials as moist sawdust, soil, peat or coir dust in wood or cardboard boxes can postpone deterioration for two to six weeks (Booth 1975; Booth 1977b; Marriot et al. 1974; Pillai et al. 1970). Another traditional storage technique, used in Kerala (India), consists of packing roots between layers of fresh leaves, preferably cassava leaves. Roots can be stored up to four weeks (Aiyer et al. 1978).

Different chemicals have been tested for postponing PPD, formaldehyde, lactic acid, ethyl bromide, calcium and sodium hypochlorite, benzoic acid, ethanol, dicloran and benomyl. Only benomyl showed a reduction of PPD up to weeks of storage (Booth 1976). Thompson and Marina Arango, cited by Rickard and Coursey (1981), found that treatment with benomyl reduced the roots surface fungal growth and treatment with chlorine reduced bacterial root rot, but neither had a delaying effect on PPD.

The use of polyethylene bags or film wraps as storage methods reduces the loss of humidity, as do traditional methods. This storage method also assures the lowering of O_2 tension as the consequence of root respiration (Rickard and Coursey 1981). However, these conditions enhance the growth of microorganisms. Therefore it is necessary to use a chemical treatment. The most recommended fungicide is Mertect (Thiabendazole) due to its non-human toxic nature (CIAT 1983; Wheatley 1985).

Curing is a natural process where wound healing is promoted under high relative humidity and temperature. After curing, cassava roots can be stored for up to four weeks (Booth 1976). The curing process induces suberisation of the outermost layers of cells at wounding sites and development of meristematic tissue (cork cambium) in the deeper parenchymatous cells, which forms a cork layer around the injury (CIAT 1974). By treating cassava roots at 35°C and 80-85% relative humidity, suberisation and cork cambium formation occurs. By raising the temperature to 40°C, meristematic tissue formation develops faster but vascular streaking occurs more rapidly, as well. The increase of relative humidity to 95% stimulates a faster microorganisms invasion, while the decrease to 75% cause the roots to dry out with no signs of wound healing (CIAT 1974).

Since metabolic processes slow down with a decrease of temperature (i.e. microbial decay, water loss, ethylene production and respiration rate), storage at low temperatures (the lowest possible without causing a chilling injury) is used to increase the storage life of perishable crops. But in tropical areas this method is limited by economical, practical and social factors (Booth 1974). Czyhrinciw and Jaffe (1951) and Montaldo (1973) reported that low storage temperatures (3-6°C) maintained cassava root quality for four weeks, nevertheless when the roots are transferred to 24°C for two days vascular streaking is observed.

As PPD has been correlated with water loss and oxygen entry to wounded roots, covering roots with paraffin wax has been used with good results. Roots can be stored up 30 days (Instituto de Investigaciones Tecnológicas 1972). This method has been successfully adopted for fresh marketing and exportation to countries where the roots can be sold at high prices.

Pre-harvest pruning of the cassava plant two to three weeks before harvest can also reduced PPD. The pruning process consists of the removal of all green material from the plant, leaving only a small stem, about 25 cm long (CIAT 1977). Pruning experiments have confirmed that PPD resistance induced by this technique lasts for at least 9 weeks after pruning and is not affected by subsequent regenerative growth (Wheatley et al. 1981). Kato et al (1991) found that the optimum period to prune cassava plants is 21-28 days before harvesting. As well, they observed that the activities of enzymes such as peroxidases and polyphenol oxidases, involved in oxidation of phenolic compunds, and phenolic compounds concentrations are minor in roots from pruned plants. Despite pruning being a feasible method in delaying PPD, it has a negative effect on root quality (i. e. dry matter content, texture, flavour). These changes are assumed to be the result of the conversion of carbohydrates reserves to sucrose, lowering the dry matter content, in order to allow leaf development and plant growth (Correa and Kato 1987, Quevedo and Bautista 1981). A recent study on the effects of pre-harvest pruning (van Oirschot et al. 2000) found that this treatment had no significant effects upon starch quality, which would make the method applicable in the starch industry.

1.6 CURRENT CASSAVA RESEARCH

Scientists around the world have pointed out diverse areas to conduct cassava research.

This research have been summarised by Scott et al (2000) in ten areas: 1) Collection and characterisation of genetic resources; 2) development of biotechnological tools for germplasm management and improvement; 3) genepool development for agro-ecologies, character improvement and variety development; 4) integrated disease management of bacterial blight, root rots, CAD, CMD and frog skin disease; 5) integrated pest management of CGM, cassava mealybug and whiteflies; 6) storage and management of planting material; 7) crop and soil management by erosion and soil fertility maintenance; 8) enterprise development, improvement in traditional processing and organisational schemes: small-large processing; 9) small-scale starch extraction processes, marketing and enterprises; 10) global characterisation and trends: characterisation of production, processing, marketing and utilisation.

1.6.1 Research on PPD

Cassava research group at University of Bath, U. K., constructed a PPD related cDNA library, from which they have isolated and characterised cDNA clones that have been involved in wound responses in other fully studied plants. The expression of the cassava catalase clone (MecCAT1) was compared between high and low PPD susceptible cultivars suggesting that high levels of catalase activity may act delaying the post-harvest occurrence (Reilly et al. 2001). A cDNA clone (cMeHRGP1) for hydroxyproline-rich glycoprotein was studied for expression during cassava storage. It starts to express from the third day of storage, when vascular streaking has already occurred, strengthening the hypothesis that wound healing is not adequate in harvested cassava roots. Other cDNA clones related with wound responses were isolated, a clone for β-1,3-glucanase, a clone for a putative serine-threonine protein kinase (cSTK1) which may be involved in signal transduction and a clone for 1-aminocyclopropane-1-carboxylate (ACC oxidase) designated as cACO1 which may help to understand the role of ethylene in PPD (Han et al. 2000; Li et al. 2000). Clones for phenylalanine ammonia lyase (PAL) and the corresponding genes expressed in PPD were also isolated (Beeching et al. 2000). Additionally, two substractive cDNA libraries from early and late stages of PPD were constructed, they will ease the isolation of more PPD related clones.

The wound responses related clones isolated from the first cDNA library constructed in Bath were screened on the mapping population at CIAT in order to determine quantitative trait loci (QTLs) and saturate the genetic map. Markers in eight linkage

groups of the female map and five linkage groups of the male map were associated with QTLs for PPD (significance level of α=0.01) (Cortes et al. in press). Research at Wageningen, The Netherlands, using cDNA-AFLP have isolated 70 transcript-derived fragments (TDFs) with the purpose of creating a catalogue of differentially expressed genes during PPD. Based on sequence homology of the 70 TDFs they identified five possible processes involved in PPD: signal transduction 12%, development 8%, stress/wound 24%, metabolism 24%, programmed cell death 6% and unknown 28% (Huang et al. 2000).

1.7 AIMS OF THE PROJECT

The aim of this project is the identification of biochemical markers for post harvest physiological deterioration, studying the occurrence of secondary metabolites produced by the activation of the phenylpropanoid pathway that may play a determinant role during PPD process. The identification of these PPD markers will generate the context and tools necessary for the improvement of cassava germplasm with respect to PPD by means of breeding and genetic modification. The finding of biochemical PPD markers will also help with the development of screening methods for use in germplasm evaluation of breeding programs.

The strategy will be based on the evaluation of cassava cultivars with different reactions to PPD. Selected biochemical tests will be used to screen the progeny of a cross between to cultivars with contrasting susceptibility to PPD, that is being used at CIAT (Centro Internacional de Agricultura Tropical) to construct the genetic map of cassava.

CHAPTER 2

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

2.1.1 Cassava Cultivars

In order to study the implication of wound responses in PPD, cassava cultivars with contrasting responses to PPD were used (Table 2.1). Cassava roots were obtained from greenhouse grown plants at Bath, harvested at CIAT and air freighted to Bath, harvested and processed at CIAT or obtained from a supermarket at Bristol.

Cultivar	Susceptibility to PPD
CM 2177-2	High
MCOL 22	High
SM 985-9	High
CM 70333-3	High
CM 523-7	High
MDOM 5	High
MNGA 2	Medium
MVEN 77	Medium
MPER 183	Low
MBRA 12	Low
MBRA 337	Low
"BRISTOL"	Medium/High

Table 2.1 Cassava (*Manihot esculenta*) cultivars with contrasting responses to PPD used in this study. The PPD susceptibility information was provided by CIAT (Dr. M. Bonierbale pers. com.). The cultivars names correspond to identification codes used by CIAT that relates to country of origin, for example MPER 183 indicates Manihot Peru accession number 183. CM refers to hybrids produced at CIAT and SM to accesions from IITA (International Institute for Tropical Agriculture, Nigeria) and retained by CIAT.

2.1.2 Evaluation of the PPD response

The PPD response has been scored for many years following the method developed by Dr. C. Wheatley (1985). After harvesting the roots were handled and transported carefully to avoid additional wounding that could interfere with the evaluation of vascular streaking. The proximal and distal ends of the root were cut and the distal end was wrapped with cling film to avoid contact with air (it blocks the physiological

deterioration). All roots were cut between 15 to 17cm long. After a storage period of three days in an open shed under ambient conditions, the post harvest damage was scored. The root was sliced transversally at 2, 4, 6, 8, 10, 12 and 14 cm from the proximal end. The seven sections were evaluated. A numeric value was given to the proximal surface of each slide. This value was based in a scale from 0 to 10. The values corresponded to 0 to 0 % of deteriorated surface, 1 to 10 %, 2 to 20 % and so on, until 10 which corresponds to 100% of deteriorated surface (Fig. 2.1). Basically the peripheral area of the portion was considered for the evaluation, because the centre rarely deteriorates. The addition of the numerical values can be expressed as percentage of deterioration, where 70 is equivalent to 100% of deterioration. For each cultivar or treatment, 10 to 20 roots were evaluated due to the high variation of the PPD response. According to the percentage of deterioration evaluated cultivars could be divided in three groups: low PPD response (0-20 %), medium PPD response (21-40 %) and high PPD response (41-100 %).

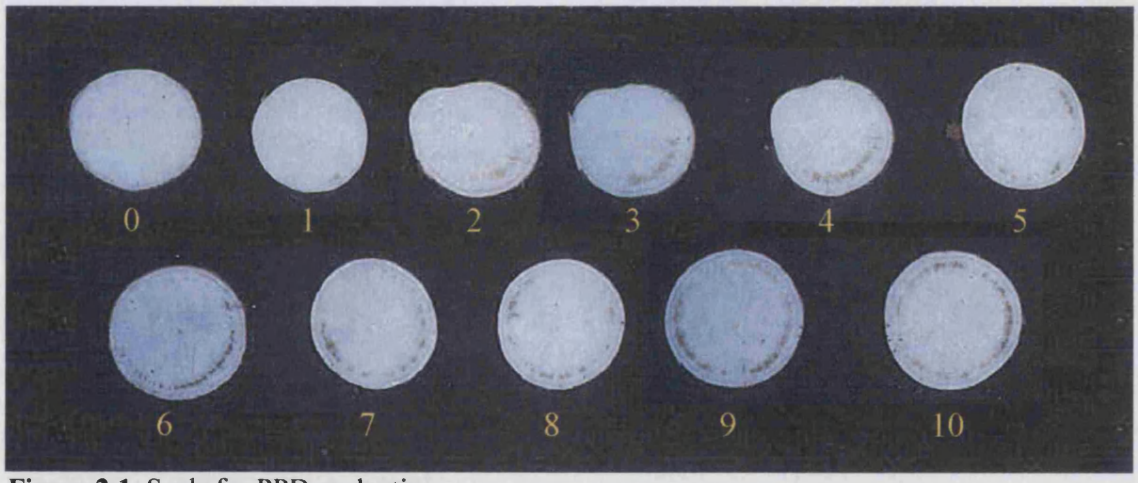

Figure 2.1 Scale for PPD evaluation

2.1.3 Harvest and Processing of Roots

The cassava roots of plants growing in the greenhouses at Bath University (temperature 22-28 °C, relative humidity 40-80 % and 14 h light period per day) were cut directly at the top of the tuberous part of the root. The roots were cleaned of adhering soil and cut into slices of same thickness. The fresh weight (FW) was determined. The slice from the upper root part was immediately extracted after harvest as described in 2.4. The other root slices were stored in a growth chamber (Fisions) at controlled conditions (dark, temperature 29 °C and relative humidity 80-90 %) during two weeks. During this

storage period, one cassava root slice was taken every day for preparing an ethanolic extract.

The cassava roots from Bristol (used for assay standardisation purposes) and those air freighted from CIAT to Bath had a special treatment to protect them during transport. After harvest the roots were cleaned of adhering soil, immersed in an anti-fungal solution (Mertek 2 % in CIAT's case) and finally covered with paraffin wax to avoid dehydration and stop post harvest deterioration. The induction of wounding stress in roots was made by cutting them into 2 cm (approx.) thick slices. Then, the periderm and the adhering wax-coating were removed. One slice was extracted immediately whereas the others were stored as described before during two weeks. One root slice was taken every day for preparing the ethanolic extracts. The cassava plants grown at CIAT were harvested eleven months after planting, this is the average harvesting time for cassava production.

The rest of the samples used in this study were harvested and processed at CIAT. In order to have a better approximation to the real events occurring during post-harvest deterioration in the field and taking advantage of the processing of roots that took place at CIAT, the wounding stress induction changed compared with the method used in the samples processed in Bath. In contrast to slicing cassava roots and storing them under constant temperature and relative humidity in growth chambers, the roots were not sliced and stored under environmental conditions. In this case, each sample comes from a whole root.

The harvesting and handling of the roots was made with extreme care to avoid additional wounding that interferes with the experimental strategy. Commercial roots of medium size, 16-18 cm minimum, without mechanical damage and microbial contamination were selected. All roots were harvested from different plants. It was made with the purpose of covering the wide range of variability that characterise cassava. After harvesting, the roots were cleaned of adhering soil with running tap water and then left to dry. The induction of wound stress was made following the same procedure that has been employed at CIAT to score PPD. All roots were cut between 15 to 17cm long. The roots were weighted and then stored in an open shed under environmental conditions. The samples were stored following a time course of seven days, sampling every day three roots per cultivar to make three replications. Before peeling off the periderm from the roots, they were weighed. This was to determine dehydration during the time course, in order to express the secondary metabolite

concentration in terms of fresh weight. Then, a one centimetre slice from each end was eliminated and the root chopped in very small cubes. One portion of the chopped root was used for preparing an ethanolic extract and the rest was frozen in liquid N_2 prior to storing at –80 °C.

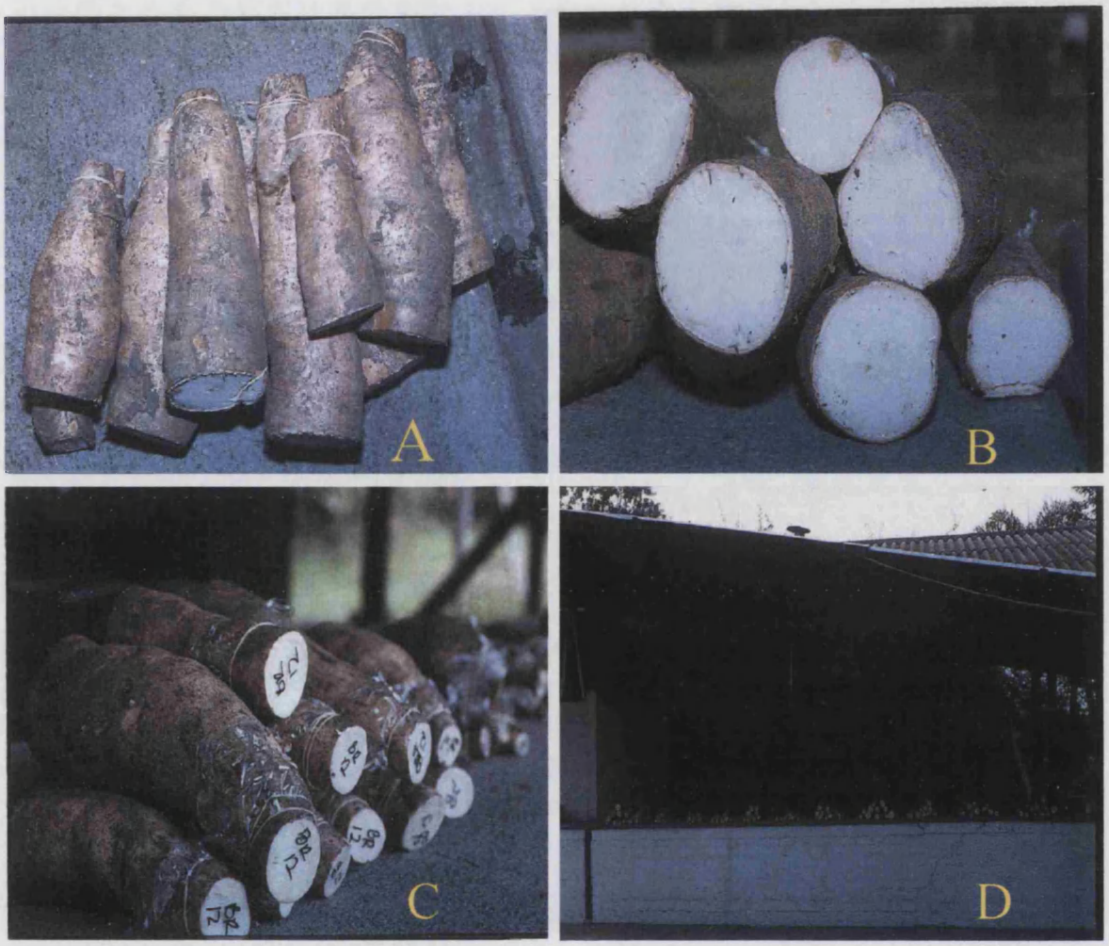

Figure 2.2 Cassava roots PPD storage at CIAT. a) Induction of wound stress by cutting the tuberous root ends. b) Roots proximal end. c) Roots distal end covered with cling film. d) Roots stored in an open shed under environmental conditions

The harvesting and processing of cassava root samples mentioned above was carried out during June 1999. Another group of samples was processed during December 1999. This group comprised samples of cultivars with contrasting responses to PPD (10 cultivars), as it was made with June 1999, and a portion (35 genotypes) of the cassava mapping population (Family K). The purpose of sampling the mapping population is the enhancement of the cassava map. The experimental design for planting the family K in the field for PPD response and other agronomical traits determination, corresponded to a partially balanced lattice design of 12 blocks, 12 genotypes per block and 20 plants per genotype. This arrangement corresponds to one replication. Three replications where planted. Owing to low availability of roots in both groups, family K

and cultivars with different responses, the sampling strategy changed. Instead of taking one complete root for each sample, roots from the same plant were cut into portions of 8 to 10 cm length and wound stressed in the same way described previously. The group of root portions coming from one plant constituted one repetition. The root portions were stored under environmental conditions following a post harvest time course of four days. The time course length was reduced in order to avoid the overlapping of physiological responses between PPD and microbial deterioration, starting four to five days after harvesting. Bearing in mind the number of roots to be processed per day (45 genotypes multiplied by three repetitions) all plants were not harvested on the same day. The experiment was divided into three groups. Each group comprises samples of the 45 genotypes, but only one plant per genotype was harvested. It means that only one repetition was processed at the same period of time. Once the post-harvest time course (four days) finished, other plant of each cultivar was harvested and processed. In that way, the three replicates were handled.

A summary of the above information respect to the different group of samples is presented in table 2.2.

Samples group	**Place of processing**	**PPD time course**	**Cultivars**
Bath	Bath	7 days	CM 2177-2, MCOL 22, CM 7033-3, SM 985-9, MDOM 5, MNGA 2, MNGA 1 and MBRA 337
June 1999	CIAT	7 days	CM 2177-2, MCOL 22, MNGA 2, MVEN 77, MBRA 12 and MPER 183
December 1999	CIAT	4 days	CM 2177-2, MCOL 22, CM 7033-3, SM 985-9, MDOM 5, MNGA 2, MVEN 77, MBRA 12, MPER 183 and MBRA 337
Family K	CIAT	4 days	35 genotypes

Table 2.2 Groups of samples assayed in this study

2.2 SECONDARY METABOLITES INVOLVED IN PPD

2.2.1 Preparation of root ethanolic extracts

After weighing the portion of chopped cassava root, it was transferred into ethanol HPLC grade (2 volumes per weight approx.) and homogenised for 60 seconds using an ultra turrax blender. The extract was incubated for one hour at room temperature and then filtered (Whatman, cellulose filter No. 1) under vacuum.

The filtered extract was transferred to a round or conical bottomed flask, adding some glass beads, and concentrated in a rotavapor at 35 °C until a volume of 1-2 ml approx. The concentrated extract was transferred to eppendorf vials and stored at –20 °C for 1 h at least. Any extract adhering to the flask walls was removed by adding a mixture of HPLC grade absolute EtOH and MilliQ H_2O (1:1) and sonicating the flask for 30 sec approx., moving the flask in a circle, allowing the glass beads to easily clean the flask walls. Then the cooled extracts were centrifuged at 12000 rpm for 10 min. Finally the supernatant was filtered through Nylon syringe filters (45 µm), transferred into brown glass sample vials and stored at -20°C until use. The cooling step has the purpose of helping to eliminate possible traces of resins in the extracts, which may cause blocking to the HPLC system.

2.2.2 Determination of the Soluble Phenol Content

For the determination of the soluble phenol content of the extracts the Folin-Ciocalteu method described by Cliffe *et al.* (1994) was used with some modifications. To 0.3 ml dH_2O and 0.5ml Folin-Ciocalteu reagent (10 fold diluted in dH_2O) 0,1 ml of extract (diluted 100 fold in dH_2O) was added. The mixture was incubated at room temperature for 6 minutes before adding 0.1 ml of saturated Na_2CO_3 (200g Na_2CO_3 in 1l dH_2O).

The absorption of the samples was measured at 755nm in a MRX Dynex Technologies microplate reader. Defined solutions of gallic acid were used as standards and the total amount of phenolics in the cassava root extracts was expressed in gallic-acid-equivalents (GAE).

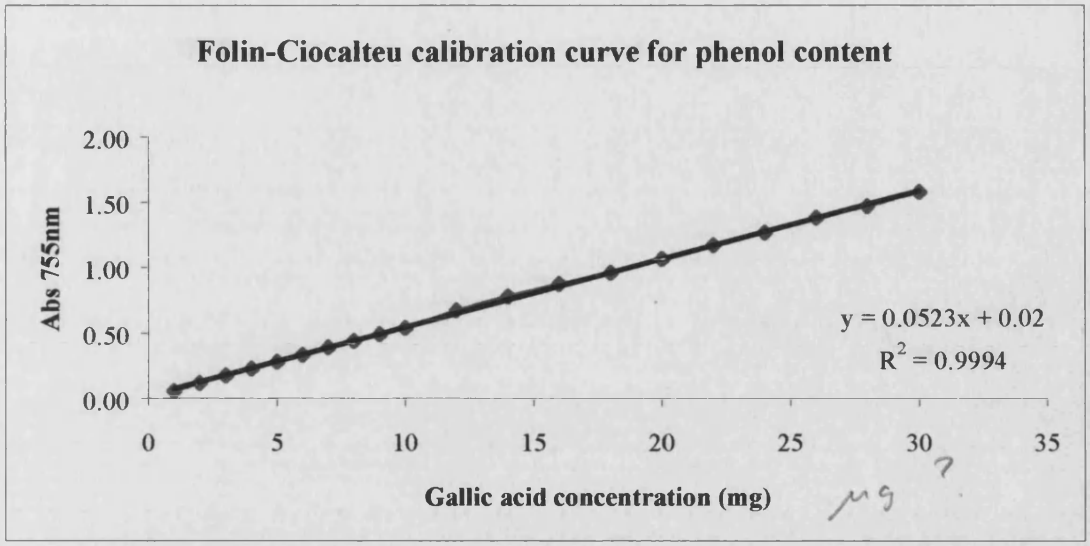

Figure 2.3. Calibration curve based on Gallic Acid for the quantification of the soluble phenol content of cassava root ethanolic extracts.

2.2.3 Chromatographic Analysis

2.2.3.1 Thin Layer Chromatography (TLC)

Before thin layer chromatography (TLC) the extracts were fractionated by liquid phase extraction, because the presence of glycosides in the crude extract blocks the migration of the metabolites in the solvent system used, which is basically non polar. A crude extract aliquot equivalent to 5 g root tissue (fresh weight) was diluted up to 6ml with ethyl acetate in a 15 ml Falcon tube. Then a 6 ml volume of MilliQ water was added. The tubes were shaken vigorously for one hour in a horizontal position to increase the contact area between the ethyl acetate and water phases. After the agitation, the tubes were left in a vertical position on the bench until the separation of the two phases, then centrifuged for 10 min at 3000 rpm to obtain a better separation. The non polar fraction (ethyl acetate) was dried, then was solved in an aliquot of ethyl acetate and stored at –20 °C until use.

Aliquots of the root extracts non polar fraction equivalent to 0.1g were spotted as 1cm lines onto HPTLC silica gel 60 F_{254} plates (Merck). The base line was drawn at 3 cm from the plate base with a soft graphite pencil to avoid scratching of the silica and interfering with the mobile phase. The solvent system used was chloroform : ethyl acetate : methanol (2 : 2 : 1).

The separated compounds were detected either by auto-fluorescence, extinction of fluorescence at 254 nm or 366 nm (CAMAG, UV lamp) or by staining. The retention

factor (R_f) of the separated compounds was determined (R_f is the value of the compound mobility based on the solvent system mobility).

For staining different reagents were used (based on Jork et al. 1990, Geissman and Griffin 1971 and Takao et al. 1994):

- **Diphenylboric acid-2-aminoethyl ester reagent (Neu reagent).** For detection of flavonoids, carbohydrates, anthocyanidines and hydroxy- and methoxycinnamic acid (Jork *et al.* 1990). The chromatograms were immersed in a solution of 1 g diphenylboric acid-2-aminoethyl ester, Naturstoffreagenz A, in 100 ml methanol, for 1 sec, dried in an oven at 100 °C for 2 min. Then the plates were immersed in a 5 % solution of polyethylene glycol 3500 in ethanol, in order to increase and stabilise the fluorescence. Finally, the plates were irradiated for 1 min with long wavelength UV light (366 nm), after drying for 1 min in an oven at 100 °C. The different metabolites produce a characteristic fluorescence in long wavelength UV light.
- **1,1-Diphenyl-2-picryl-hydrazyl.** For detection of antioxidant metabolites. The developed TLC plates were immersed for 1 sec in a solution of 5 mg of 1,1-diphenyl-2-picryl-hydrazyl, DPPH free radical, in 100 ml methanol. Then the plates were dried in an oven at 100 °C for 1 min. Clear bands on a pink-light purple background reveal the presence of antioxidant metabolites.
- **Antimonium III chloride.** For detection of terpenoids. The TLC developed plates were immersed in a solution of 1 % antimonium III chloride in chloroform for 1 sec. Then the plates were dried in an oven for 1 min. Terpenoids were visualised under long wavelength UV light (366 nm). These metabolites present a characteristc pink-red fluorescence.

2.2.3.2 High Performance Liquid Chromatography (HPLC)

For better separation (compared to TLC), for quantification and for isolation of the components of cassava root extracts, reversed phase high performance liquid chromatography (RP-HPLC) was used in analytical or preparative scales.

The following material and parameters were used for the HPLC:

	Analytical HPLC	**Preparative HPLC**
Stationary Phase and Column Dimensions	HPLC TECHNOLOGY, Techsphere ODS BDS; 5µm particle size, 250 x 4.6mm	HPLC TECHNOLOGY, Techsphere ODS BDS; 5µm particle size, 250 x 10mm
Pumps	GILSON 302, 303 and 305	GILSON 305

Injector	GILSON 231 sample injector or RHEODYNE 7125 manual injector	RHEODYNE 7125 manual injector
Detector	GILSON 116 UV-VIS detector or HP 1040a diode array detector	GILSON 116 UV-VIS detector
Detection Wavelength	280nm and 350nm (single wavelength mode or simultaneously)	280nm
Control and Evaluation Software	GILSON 715 or HP HPLC ChemStation	GILSON 715

Table 2.3 Materials and parameters used for analytical and preparative HPLC.

The compounds in the HPLC chromatograms were identified by comparison of retention times, by co-chromatography and by comparison of UV spectra (collected by the diode array detector at 190-400 nm) with those of identical reference compounds. Reference compounds were obtained from Sigma. Scopolin was kindly provided by Professor Goro Taguchi, Shinshu University, Japan.

For analytical runnings, as mobile phase, a gradient system of acetonitrile (CH_3CN) and MilliQ water plus H_3PO4 (pH 2.6) under the following parameters was used:

Time (min)	CH_3CN (%)	Flow Rate (ml/min)
0 – 5	2	1.0
5 – 20	10	1.0
20 – 40	30	1.0
40 – 55	65	1.0
55-56	100	2.0
56-61	100 (washing)	2.0
61-64	2	1.0
64-70	2 (equilibration)	1.0

Table 2.4 Mobile phase used for analytical HPLC

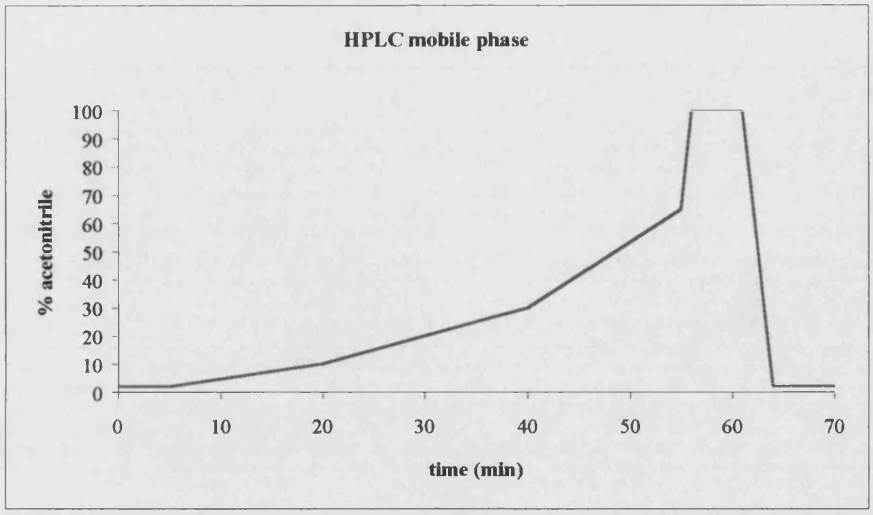

Figure 2.4 Acetonitrile-H_2O gradient solvent system in terms of acetonitrile percentage.

For preparative purposes a linear gradient of MilliQ-H_2O plus H_3PO_4 (pH 2.6) to CH_3CN (100 % to 0 %) over a period of 60 min and a flow rate of 6 ml/minute was used. The injection volume ranged from 200 to 500 µl of extract. The separated peak-fractions were collected in glass tubes by using a Gilson 203 fraction collector. As a control, 50 to 100 µl of the fractions were re-injected into the HPLC using the analytical parameters. The isolated fractions were dried and stored until further use at –20 °C.

Before preparative HPLC some of the extracts were fractionated using solid phase extraction (SPE) on a vacuum manifold (Whatman). For this, the SPE columns (IST, ODS-C_{18} columns) were activated by washing with 3 ml methanol and 3 ml H2O. 500 µl of the extracts were applied onto the columns and washed with 5 ml of H_2O afterwards to collect the "polar" fraction. The ODS material retained "non polar" components which were recovered by washing with 5ml acetonitrile (CH_3CN). Both fractions were dried, then resuspended in an aliquot of H_20, polar fraction, or CH_3CN, non-polar fraction, and stored until use at –20 °C.

2.2.4 Spectroscopic Analysis

In order to obtain more precise information on the nature and identity of the secondary compounds present in cassava root extracts, spectroscopic methods such as UV and mass spectroscopy (MS) were used. Those spectroscopic methods in combination with liquid chromatography (LC) have been employed as a very useful tool in the rapid identification of secondary metabolites (Renukappa et al. 1999).

The dried peak fractions from preparative chromatography and some crude ethanolic extracts were sent to Mrs. Iris Klaiber, under the supervision of Dr. Bernard Vogler at the Institute of Chemistry, University of Hohenheim (Germany), for running LC-MS. Those experiments were carried out on a Finnigan TSQ 700 under atmosphere pressure chemical ionisation (APCI), ion source=APCI + Q1MS and LC gradient 2-65 % CH_3CN in 55 min as described by Vogler et al. (1998).

2.2.5 Determination of tannin content in cassava root tissue

The determination of the tannin content of cassava root tissues undergoing PPD was made following the acid hydrolysis protocol used by Rickard (1986) with some modifications.

Ground freeze dried cassava tissue was used instead of fresh tissue in order to avoid possible interferences of H_2O in the proper detection of tannins. 0.25 g of ground freeze dried cassava tissue was added to 10ml HCl in butanol (5 % v/v). The solution was heated at 95 °C for 90 min, followed by centrifugation at 5000 x *g* for 20 min. Finally the absorbance of 1 ml of the supernatant was read at 550 nm in a Cecil spectrophotometer, against an unheated sample blank prepared in the same way. Defined solutions of tannic acid were used as standards and the total content of tannin in the cassava root extracts was expressed in tannic acid equivalents (TAE).

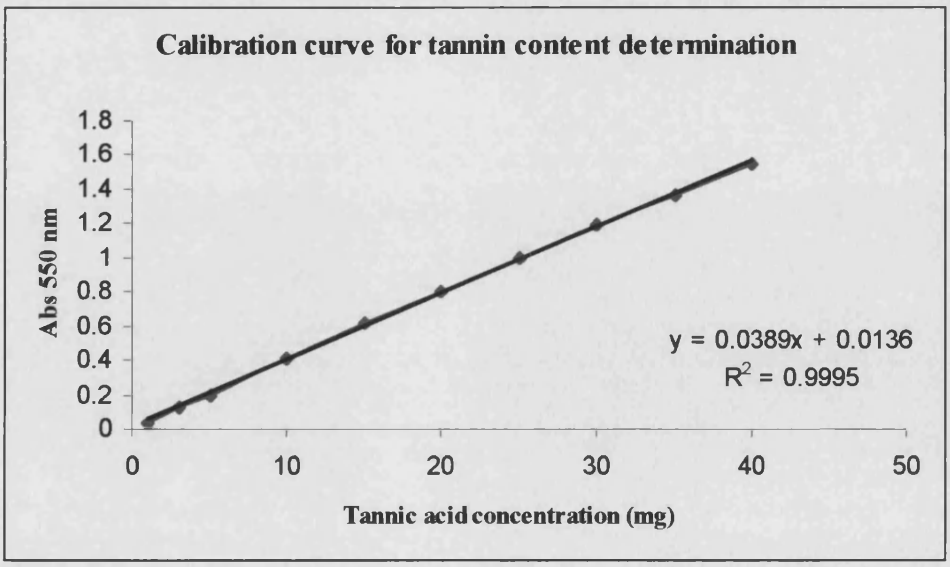

Figure 2.5. Calibration curve based on tannic acid for the quantification of the tannin content cassava roots.

2.2.6 Localisation of secondary metabolites (lignin, callose, suberin and flavonoids)

Following a PPD time course of four days, root slices were cut transversally (1-2 mm thick approx.) with a sharp knife, and root sections for light microscopy were hand cut with a razor blade and placed on dH_2O just after cutting to prevent dehydration and eliminate starch. After staining, the root sections were placed on glycerol for microscopic observation.

2.2.6.1 Staining for lignin

Root tissue cuts were dipped in 0.1 % phloroglucinol in 20 % HCl for 10 min. Then the tissue sections were washed with dH_2O and 70 % EtOH. Lignified tissue turns deep red.

2.2.6.2 Staining for callose

Tissue sections were immersed in 0.01 % aniline blue in potassium phosphate, pH 9.5, during 15 min. The stained tissue was observed under long UV light. Callose presents a yellow fluorescence.

2.2.6.3 Staining for suberin

Tissue slices were stained in a saturated Sudan III solution (0.5 %) in 70 % EtOH during 6 min. Then the tissue was washed with 70 % EtOH. Suberised tissue stains deep red.

2.2.6.4 Staining for flavonoids

Root tissue portions were immersed in 1 % diphenylboric acid-2-aminoethyl ester, Neu's reagent, in MeOH for 5 min. The flavonoids are observed as yellow-orange fluorescent spots under long UV light.

2.3 PPD RELATED ENZYMATIC ASSAYS

The chopped cassava root tissue stored at –80 °C was transferred to a mortar and first ground with a pestle to destroy the big clusters. Then liquid N_2 was added to the sample to keep it frozen and make sure a better grinding of the tissue. After that, the frozen cassava root tissue was ground in a food processor until a very fine powder. Finally 5 g

of the ground sample were transferred to 50 ml Falcon centrifuge tubes to be used in the extraction of the different enzyme activities tested.
All enzyme activity reactions were performed in triplicate.

2.3.1 Assays for enzymatic activity

2.3.1.1 Phenylalanine ammonia lyase (PAL)

The activity of PAL was measured spectrophotometrically by the formation of cinnamic acid, following the protocol proposed by Edwards and Kessmann (1992) with some modifications.

Enzyme extraction. Ground cassava root tissue (5 g) was suspended in 10ml of ice-cold extraction buffer, containing 50 mM Tris-HCl pH 8.5 and 14 mM 2-Mercaptoethanol. Polyvinyl polypyrrolidone, PVPP 5 % (w/w), was added to the mixture to adsorb phenols. After homogenisation with extraction buffer, the samples were shaken on ice for 1 h. The tubes were placed parallel to the rocking table surface to assure a larger shaking area (the same procedure was followed for the different enzyme extractions). Following shaking, extracts were centrifuged at 2000 x *g* for 15 min at 4 °C and the supernatant centrifuged again at 12000 x *g* for 20 min at 4 °C. Then, 2.0 ml of the supernatant were passed through a sephadex G25 column (PD-10 Pharmacia), pre-equilibrated with Tris-HCl buffer 50 mM pH 8.5 at 4 °C, in order to eliminate low molecular weight compounds. The filtered extract was used for the activity assay.

Activity assay. The activity assay was performed in a total volume of 1 ml. The reaction mixture comprised 0.1 ml of the enzyme extract, 50 mM Tris-HCl pH 8.5 and 10 mM L-phenylalanine. The enzymatic reaction was incubated at 40 °C, while a control reaction was performed in the same conditions but using D-phenylalanine as substrate. The formation of cinnamic acid was followed by absorbance readings at 30 min intervals in quartz cuvettes at 290 nm wavelength during 2 h.

2.3.1.2 Peroxidases (POX)

The procedures proposed by van der Berg et al. (1983) and van Gestelen 7. (1998) were followed with some modifications.

Enzyme extraction. Ground cassava root tissue (5 g) was suspended in 10 ml of cold extraction buffer, comprising 50 mM potassium phosphate buffer pH 7.0, 1 mM Na-EDTA, 1 mM dithiothreitol, DTT (to prevent oxidation) and 5 % (w/w) polyvinyl polypyrrolidone, PVPP (to adsorb phenols). Following shaking on ice for 1 h, extracts

were centrifuged at 2000 x *g* for 15 min at 4 °C and the supernatant centrifuged again at 12000 x *g* for 20 min. Then, 2 ml of the supernatant were filtered through a sephadex G25 column, pre-equilibrated with potassium phosphate buffer 50 mM pH 7.0 plus 0.5 mM Na-EDTA at 4 °C, in order to separate the protein from low molecular weight compounds. The filtered extract was used for the activity assay.

Activity assay. Peroxidase activity was assayed spectrophotometrically by the oxidation of 3,5-dichoro-2-hydroxy-benzenesulfonic acid (DHBS), which in its oxidised form establishes a coloured complex with 4-aminoantipyrine (AA) that absorbs at 510 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.0), 10 mM DHBS, 1 mM AA, 10 mM H_2O_2 and 100 µl enzyme extract in a total volume of 3 ml. The reaction was started by adding H_2O_2, and the change in absorbance at 510 nm was measured using a Beckman spectrophotometer, during five minutes.

2.3.1.3 Scopoletin peroxidase (SCP-POX)

The protocol used was based on the procedures proposed by Gutierrez *et al.* (1995) and Edwards *et al.* (1997).

Enzyme extraction. The same extract used for the peroxidase assay was used for the activity of scopoletin peroxidase.

Activity assay. Scopoletin peroxidase activity was measured spectrophotometrically by the oxidation of scopoletin, which changes to a dark blue-green colour. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 0.1 mM scopoletin (dissolved in ethanol), 1 mM H_2O_2 and 10 µl enzyme extract in a total volume of 1 ml. The reaction was started by the addition of H_2O_2, and the continuous oxidation of scopoletin was monitored at 395 nm in a Beckman spectrophotometer during four minutes.

2.3.1.4 Catalase (CAT)

The methods proposed by Escobar *et al.* (1996) and van Gestelen pers. com. were followed with modifications.

Enzyme extraction. The extract used for the peroxidase assay was used for the activity of catalase.

Activity assay. Catalase activity was measured by the break down of H_2O_2 into H_2O and O_2. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM

Na-EDTA. 0.05 % w/v H_2O_2 and 500 µl enzyme extract in a total volume of 3 ml. The reaction was started by addition of H_2O_2, and the continuous decrease in absorbance at 240 nm was measured (during five minutes), in quartz cuvettes.

2.3.1.5 Polyphenol oxidase (PPO)

Several extraction and activity protocols were assayed without good results. Some times there was no reaction or the linearity of the reaction was not acceptable. Extraction buffers with different salts such as sodium phosphate, potassium phosphate, sodium acetate, Tris and tricine, and pH from 5.5 to 8.0 were tested. As well, a wide range of substrates was assayed. The phenolic compounds used were L-Dopa, chlorogenic acid, cafffeic acid, and catechol, in combination with different assay buffers with pH from 4.0 to 8.0. Finally the activity of PPO was determined by the oxidation of (+)-Catechin following the protocol proposed by Data et al. (1984) with some modifications.

Enzyme extraction. Ground cassava root tissue (5 g) was suspended in 10 ml of cold extraction buffer, containing 50 mM potassium phosphate buffer pH 7.0, 1 mM Na-EDTA, 0.1 % sodium ascorbate (as antioxidant) and 5 % (w/w) polyvinyl polypyrrolidone, PVPP (to adsorb phenols). After 1 h shaking on ice, extracts were centrifuged 2000 x g for 15 min at 4 °C and the supernatant centrifuged again at 12000 x g for 20 min. Then 2 ml of the supernatant were filtered through a sephadex G25 column, pre-equilibrated with potassium phosphate buffer 50 mM pH 7.0 plus 0.5 mM Na-EDTA at 4 °C, in order to separate the protein from low molecular weight compounds. The filtered extract was used for the activity assay.

This extraction method was used for the first group of enzymatic assays (June 1999). Then with the second group of enzyme assays (December 1999), the extraction method was changed for the peroxidase method in order to simplify and gain time, having in mind the large amount of samples.

Activity assay. The reaction mixture contained 0.8 ml of 100 mM potassium phosphate buffer (pH 6.0), 50 µl of aerated 5 mM (+)-catechin, dissolved in assay buffer, and 0.25 ml enzyme extract. The mixture was incubated at 30 °C for 30 min. The reaction was stopped by adding 0.4 ml of 2 N $HClO_4$. Finally the stopped reaction was centrifuged at 5000 rpm during 10min. The absorbance of the oxidised catechin was read at 395nm.

2.3.1.6 B-1,3-Glucanase and chitinase

Glucanase and Chitinase were assayed by the release of reducing sugars from the relevant substrate by the reaction of Somogyi-Nelson (Somogyi 1951).

Enzyme extraction. Ground cassava root tissue (10 g) was suspended in 10 ml of ice-cold extraction buffer, 50 mM sodium acetate pH 5.0 and 5 % (w/w) polyvinyl polypyrrolidone, PVPP. After 1 h shaking on ice, extracts were centrifuged at 2000 x *g* for 15 min at 4 °C and the supernatant centrifuged again at 12000 x *g* for 20 min. Then 5 ml of the supernatant were precipitated with $(NH_4)_2SO_4$ to 80 % of saturation at –20 °C over night, in order to remove reducing sugars. The high amount of glycosides present in cassava roots makes the determination of reducing sugars released by the hydrolytic enzyme activity difficult. The precipitate was collected by centrifugation at 20000 x *g* for 30 min and suspended in 50 mM sodium acetate pH 5.0. The suspended protein was applied to a size exclusion chromatography column, sephadex G25 (PD 10, Pharmacia), to remove salts and other low molecular weight components. The column was pre-equilibrated with 50 mM sodium acetate buffer pH 5.0 at 4 °C.

Activity assay. The procedure proposed by Fisher *et al.* (1989) was modified to perform the assay. Activity reactions for both enzymes were performed in the same way. A solution of 0.5 % of laminarin from *Laminaria digitata* in 50 mM sodium acetate buffer pH 5.0 was used as substrate for β-1,3-glucanase, and 5 mg/ml solution of colloidal chitin[1] in 100 mM sodium acetate buffer pH 5.0 for chitinase. 50 µl of enzyme extract were transferred to a 2 ml screw cap eppendorf vial. Then, an aliquot of 200 µl of the corresponding substrate, previously warmed at 37 °C, was added to the enzyme. After homogenisation, the reaction mixture was incubated at 37 °C during 30 min. In the case of chitinase the vials were shaken occasionally during the incubation period, because the chitin tends to precipitate to the eppendorf tube bottom. After the incubation, the reaction was terminated adding 200 µl of reagent Somogyi I[2] and 50 µl of reagent

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[1] Colloidal chitin preparation. The method proposed by Skujins *et al.* (1965) was followed with some modifications. Stir 10g of chitin, technical grade, in 100ml H_3PO_4 concentrated (85%) for 48h at 4°C. Then, filter the resulting thick liquid through glass wool and stir vigorously into a 50% aqueous ethanol solution. After the stirring, wash the precipitate, chitin in a highly dispersed state, with dH_2O several times to remove the excess of ethanol and acid. Dialyse the precipitate against dH_2O for 48h to remove chitin oligosaccharides and low molecular weight impurities. Finally, dilute the suspension to 10mg/ml (chitin dry weight) with dH_2O and store at 4°C with a crystal of thymol to prevent contamination.

[2] Somogyi-Nelson reaction solutions preparation.
Somogyi I. Dissolve in dH_2O up to one litre, 24g Na_2CO_3 anhydrous, 16g $NaHCO_3$, 12g Rochelle salt and 144g Na_2SO_4. The solution tends to precipitate, in that case, warm it up before using
Somogyi II. Dissolve in dH_2O up to 200ml, 4g $CuSO_4.5H_2O$ and 36g Na_2SO_4.

Somogyi II. Caps were placed on the tubes and incubated at 95 °C, in a water bath, for 30 min to develop the coopper complex. Following cooling of the tubes and vortexing, 250 µl of Nelson reagent and 700 µl dH_2O were added. Finally the tubes were left opened over night, in the dark, to liberate the CO_2 produced by the reaction with Nelson reagent. 200 µl of the reaction were transferred to flat bottom microplates and the absorbance read at 595 nm in a MRX Dynex Technologies microplate reader. Blanks and time zero controls were treated in the same way as activity reactions. In order to convert the absorbance values into units of monosaccharides liberated by the reaction, standard curves of glucose for β-1,3-glucanase and N-acetyl glucosamine for chitinase were produced.

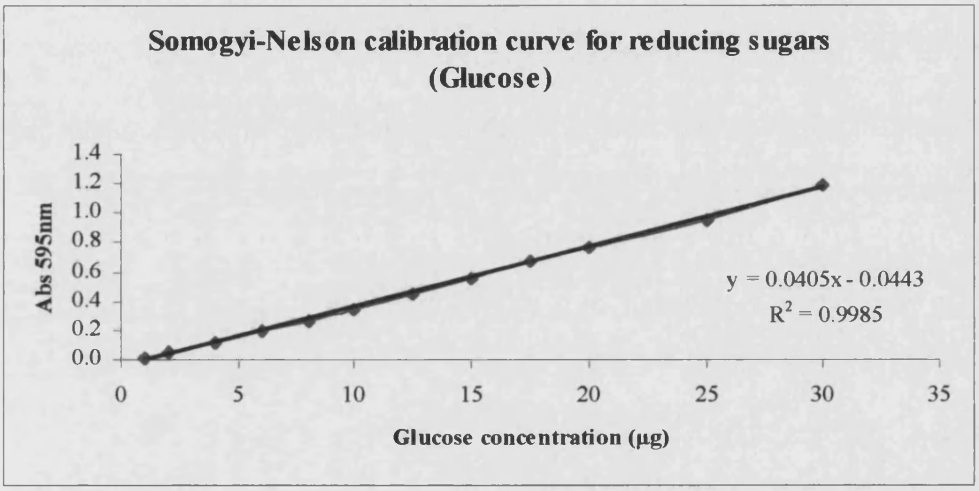

Figure 2.6 Calibration curve for Somogyi-Nelson reaction in terms of Glucose.

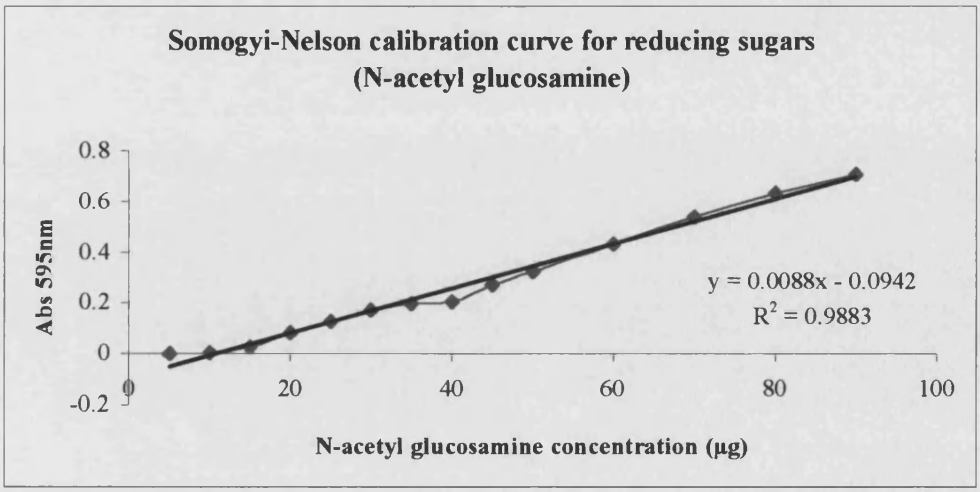

Figure 2.7 Calibration curve for Somogyi-Nelson reaction in terms of N-acetyl glucosamine.

Nelson. Stir 25g of ammonium molybdate in 450ml dH_2O, then add 21ml of H_2SO_4. Mix until it is well dissolved. Apart, dissolve 3g of $NaHAsO_4.7H_2O$ in 25ml dH_2O. Mix both solutions and incubate for 48h

2.3.1.7 Total protein quantification

The total protein quantification of the enzyme extracts was made following the method proposed by Bradford (1976). 20 µl of protein extract was mixed with 50 µl of 1.5 M NaOH and 1 ml Bradford reagent[3] in a 1.5 ml eppendorf tube. The reaction was incubated for 5 min at room temperature. Then, 200 µl of the reaction were transferred to a flat bottom microplate. Readings were made at 595 nm in a MRX Dynex Technologies microplate reader. The absorbance readings were then converted into concentrations of micrograms of protein per microlitre by a calibration curve from known concentrations of bovine serum albumin (Fig. 2.8.). The logarithm (log_{10}) of the protein concentration and absorbance (595 nm) was used to construct the calibration curve, in order to obtain a linearization of the relation curve between protein concentration and Bradford reagent absorbance which is not linear (Stoscheck 1990). For each protein extract three independent replicates were made.

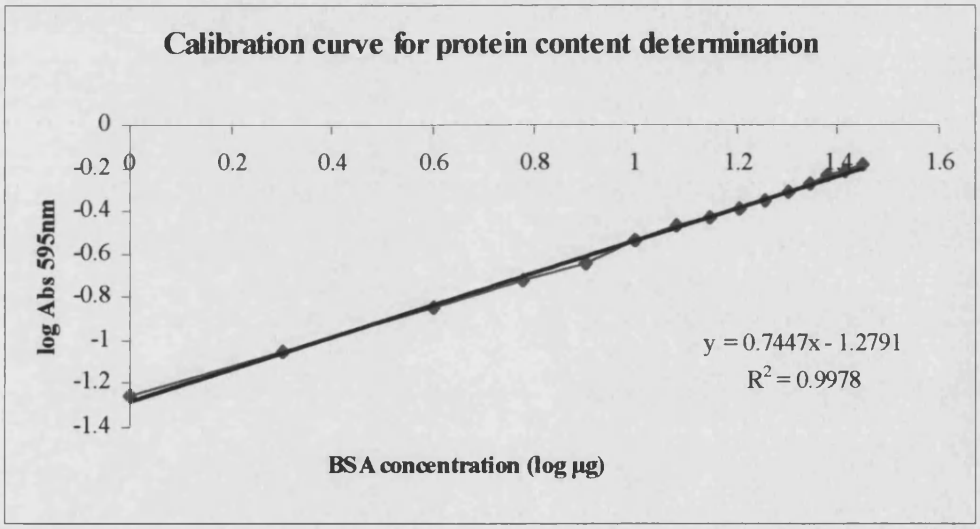

Figure 2.8 Calibration curve for protein quantification based on Bradford reaction with bovine serum albumin (BSA) as a protein standard.

2.3.2 Localisation of Reactive Oxygen Species (ROS) and Enzyme Activity by Tissue Printing

Post-harvest stress was induced by cutting the proximal and distal ends of the root, which were covered with cling film, additionally two V shaped incisions (opposite one to the other) were made through the periderm along the length of the root. This

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at 37ºC. Store in a dark container.

[3] Bradford reagent preparation. Stir 100mg of Coomasie brilliant blue G with 100ml H_3PO_4 concentrated (85%) and 50ml EtOH (95%) for several hours at room temperature. Then, dissolve the mixture slowly

wounding procedure was used with the purpose of taking all the samples of the time course from the same root; in this case it might be easier to monitor the change of enzymes and ROS localisation. Root tissue portions were sampled following a time course of four days. Every day, root portions were extracted from the distal and proximal ends, and root tissue slices were hand cut transversally with a very sharp knife.

Figure 2.9 Wounding stress by V shaped incisions along the cassava root.

2.3.2.1 Localisation of reactive oxygen species (H_2O_2)

A modification of the Vallelian-Bindschelder *et al.* (1998) method was used for hydrogen peroxide localisation in root tissue.

Hand cut root slices, 1-2 mm thick approx., were vacuum infiltrated with 2 mg/ml 3,3 diaminobenzidine tetrahydrochloride (DAB) and incubated at room temperature for 3h. As a control, root slices were infiltrated with 2 mg/ml DAB and 10 mM sodium ascorbate, a H_2O_2 scavenger. Immediately after incubation, root slices were washed with dH_2O and documented by direct scanning.

2.3.2.2 Tissue print localisation of enzyme activity

Tissue prints were made on nitrocellulose membrane, placed on 3MM Whatman filter paper, by pressing softly the root slice onto the membrane during 30 sec. The pressure was not made directly with fingers. A glass slide was used to press directly on top of the root tissue to guarantee a homogeneous force all over the tissue area. The prints were left on the bench for some minutes to let them dry. After the treatment for localisation of the different enzymes the coloured tissue prints were documented by direct scanning.

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with ddH_2O until 1 litre. Allow the solution to precipitate over night at 4°C, and centrifuge at 11000xg at

2.3.2.2.1 Tissue print localisation for peroxidase

Detection of peroxidase activity was made following the protocol proposed by Peyrado *et al.* (1996). The printed nitrocellulose membrane was soaked in 50 mM potassium phosphate buffer, pH 5.3, and 0.1 % H_2O_2 in 10mM aqueous guaiacol for 5 min approx., until a dark brown coloration was developed. Alternatively, the peroxidase activity was localised by reaction with 3,5-dichoro-2-hydroxy-benzenesulfonic acid (DHBS) and 4-aminoantipyrine (AA), using the same conditions as the enzyme activity assay previously described.

2.3.2.2.2 Tissue print localisation for scopoletin peroxidase

The printed membrane was immersed in 0.1 M potassium phosphate buffer (pH 6.5), 0.1 mM scopoletin and 1 mM H_2O_2, approximately during 5 min until a bright blue-purple coloration was developed.

2.3.2.2.3 Tissue print localisation for polyphenol oxidase

The nitrocellulose prints were dipped in 0.1 M potassium phosphate buffer (pH 6.0) and 0.5 mM (+)-catechin (previously aerated) during 5 h until a bright orange coloration was developed. The incubation was performed in the dark to prevent substrate light oxidation.

2.3.2.2.4 Tissue print localisation for hydroxyproline rich glycoproteins (HRGPs)

Nitrocellulose prints were soaked for 1 h, under shaking at room temperature, in 5 % powdered skim milk solution prepared in PBS[4] buffer. Afterwards, the membranes were washed for 30 sec, three times, with dH_2O. Once the washings became clear, the membranes were covered with 2 ml, per membrane approx, 1/10 diluted primary antibody in PBS; and then the container was covered with cling film. The primary antibody, LP10 from carrot, was kindly provided by Dr. Helen Thomson. After incubation on a shaker for 1 h at room temperature, the membranes were washed with dH_2O, six times. After the washings, the membranes were incubated with approximately 5 ml of secondary antibody (Anti-Rat IgG, peroxidase conjugate, SIGMA), 1/200 diluted in PBS, as with the primary antibody. Following incubation,

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4°C. The reagent is stable for six months in the dark at 4°C.

[4] PBS buffer

Dissolve in dH_2O up to 500ml 40g NaCl, 1g KCl, 1g KH_2PO_4 and 14.3g K_2HPO_4. Adjust pH to 7.2. Autoclave the solution and store at -4°C.

the membranes were washing again six times with dH_2O. Finally, in order to visualise the HRGPs, membranes were covered with 4-cholo-1-naphthol standard solution[5] during 20min approximately, until a light lavender coloration was developed. After that, the membranes were washed four times with dH_2O. Then, the membranes were placed on absorbent towels to let them dry and documented by photography.

2.3.3 Western blot for hydroxyproline rich glycoproteins (HRGPs)

In order to test for the presence of HRGPs in cassava root tissues undergoing PPD and eliminate possible artefacts in the tissue printings, a western blot for soluble HRGPs was performed.

The protein extracts were run in polyacrylamide gels[6] (10 % acrylamide and 5.6 % stacking). 10 µg protein were mixed with Laemmli[7] dissociating solution in 1:1 proportion, heated at 100 °C for 2 min, cooled on ice and finally centrifuged at 5000 rpm for 10 min. Then the gels were run for 2 h at 100 V. The protein transfer was carried out in a Mini Trans-Blot, Electrophoretic Transfer Cell (Bio-Rad), at 100 V for 1 h. The transfer cell was placed on ice during the blotting in order to keep buffer[8] and cell low temperature. After transfer the nitrocellulose membranes were washed three times for 5 min with 1x TSBT[9]. Then the membranes were treated as previously described for tissue prints, increasing the solutions volume having in mind the blots size.

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[5] 4 chloro-1-naphthol standard solution
Mix 8ml 0.48mM 4 chloro-1naphtol with 48µl 6% H_2O_2.

[6] Polyacrylamide gels preparation (for two small gels)
Polyacrylamide gel 10%
Mix in this order 4.02ml dH_2O, 5.18ml 1M Tris pH 8.8, 140µl 10% SDS, 4.66ml Acrylamide 30%/Bis0.75%, 6µl Temed and 60µl 10% APS.
Stacking gel 5.6%
Mix in this order 4.9ml dH_2O, 5.18ml 1M Tris pH 6.8, 1.3ml Acrylamide 30%/Bis0.75%, 70µl 10% SDS, 6µl Temed and 60µl 10% APS.

[7] Laemmli Dissociating Solution
Mix 2.5ml dH_2O, 1ml 1.25M Tris pH6.8, 1ml 10%SDS, 1.2ml β-mercaptoethanol, 0.5g sucrose and 0.1% Bromophenol blue.

[8] Western blot transfer buffer
Dissolve in dH_2O up to 1l 3.02g Tham, 14.4g Glycine and 200ml EtOH.

[9] 5X TSBT
Dissolve in dH_2O up to 1l 12.11g Tham, 43.83g NaCl and 2.5ml Tween 20. Adjust pH to 7.2.

2.3.4 Isoelectric focusing (IEF) for peroxidase and scopoletin peroxidase

With the aim of finding more marked differences between cassava cultivars with contrasting responses to PPD, IEF was applied to protein extracts.

2.3.4.1 Enzyme extraction

The procedure followed was the same of the extraction for peroxidase activity assay, but using 10 g of ground root tissue instead of 5 g because it was necessary to work with a very concentrated protein extract to detect activity bands on the IEF gels. Besides using more root tissue, another 2 ml of crude extract were filtered through the sephadex G25 columns. After desalting the extracts, the filtrates were concentrated. The filtrated extracts (approx. 6 ml per sample) were transferred to cellulose dialysis tubing, previously cleaned, and placed over a layer of polyethylene glycol (PEG) in a tray. The dialysis bags were covered, as well, with PEG to accelerate the concentration process. The samples were reduced to 1 ml volume approx. The PEG concentration procedure was carried out at 4 °C in order to avoid enzyme activity degradation.

2.3.4.2 Isoelectric focusing

The concentrated protein extracts were run on an Ampholine PAG polyacrylamide gel (Amersham Pharmacia), pH range 3.5-9.5, in a Bio-Phoresis® horizontal electrophoresis cell (Bio-Rad). The electrophoresis cell was connected to a circulator thermostatic water bath, which was turned on at 10 °C 30 min before loading the protein samples on the gel. A small volume of 1 M NaOH was poured in the cavities located at each side of the electrophoresis machine with the purpose of trapping CO_2, which may have an effect on the pH of the electrode buffers during the electrophoresis run. Before placing the IEF gel on the cooling platform, 2-3 ml of Bayol-F oil were drizzled over the plate. Then one edge of the gel was placed on the cooling platform and laid down slowly with the intention of creating a very thin film of oil and to avoid air bubble formation. The oil film enhances the heat transmission to the platform and air bubbles cause disruptions in the protein migration. Once the gel was properly positioned, the plastic gel cover was carefully removed. Electrode strips were prepared by saturation with 3 ml, approximately, of the corresponding solutions, 0.1 M NaOH for the cathode and 0.04 M aspartic acid for the anode. 20 µg of protein were served onto the sample application papers. The gel was first run at 1500 V, 50 mA and 30 W for 45 min, then the sample

application papers were removed and the gel run for 30 min more under the same conditions.

After running the peroxidase and scopoletin peroxidase isoforms were revealed by immersing the gels in the buffers and substracts previously described for the enzymatic activity assay. As soon as the coloration was developed the gels were photographed. As isoelectric point marker, the IEF standards matker (pH 4.45-9.6) from Bio-Rad was used. Before developing the isoforms, the gel line corresponding to the pI marker was cut and then stained with Coomassie blue R solution[10] for 2h, cleaned with distaining solution I for 2 h and distaining solution II for 30min.

2.4 BIOLOGICAL ACTIVITY OF SECONDARY METABOLITES PRESENT IN CASSAVA ROOTS DURING PPD

The anti-microbial activity of the secondary metabolites present in cassava roots undergoing PPD was detected by different bioassays.

2.4.1 Micro-organisms

The bioassays were performed using conidial suspensions of *Trichoderma harzianum*, which has been related with post-harvest decay or microbial deterioration (Wenham 1995), and *Fusarium avenaceum* and *Cladosporium cucumerinum* that do not have links with cassava diseases but have been widely used for test the biological activity of naturally occurring compounds. The fungi were grown on malt agar[11] for five days before spraying the conidial suspension on TLC plates. To test the antibacterial activity of cassava root extracts *Staphylococcus aureus* was used, as with *F. avenaceum* and *C. cucumerinum,* these are not associated with cassava.

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[10] Solutions for pI marker staining
Staining stock solution
Dissolve 2g Coomassie Blue R-250 in 200ml dH_2O.
Staining solution
Mix in dH_2O up to 500ml 62.5ml staining stock solution, 250ml Methanol and 50ml Acetic acid.
Destaining solution I
Mix in dH_2O up to 1l 500ml Methanol and 100ml Acetic acid.
Destaining solution II
Mix in dH_2O up to 500ml 25ml Methanol and 35ml Acetic acid.

[11] Malt Agar
Dissolve 20g Malt extract in dH_2O and add 18g agar. Autoclave at 121°C for 15min

2.4.2 Sample Preparation by Fractionating

In some TLC runs it was observed that the ethanolic crude extract did not resolve properly in the solvent system. Therefore, the crude extract was separated into its polar and non-polar fractions by solid phase extraction (SPE) or liquid phase extraction (LPE). SPE was performed as previously described. For LPE one aliquot of the crude extract was diluted to 15 ml with MilliQ H_2O, then 15 ml butanol, a non polar solvent, was added to the water dilution and mixed constantly by inversion for 5 min. The mixture was left at room temperature until the two phases separated. The butanolic phase was carefully removed. 15 ml of ethyl acetate, a medium polar solvent compared to butanol, was mixed with the water phase and the same procedure as with butanol was followed. Finally the three phases were dried and resuspended in a small volume of the corresponding solvent and stored at –20 °C.

2.4.3 Thin Layer Chromatography (TLC) Bioautography

Aliquots of the medium and non polar fractions corresponding to 1 g to 4 g fresh weight were spotted onto normal phase TLC plates, including one sample of the different solvents as a control. After the evaporation of solvents, the plates were developed in the solvent system previously mentioned. Once the plates were dried, the bands were determined under UV light. The polar fractions were just spotted onto the TLC plates, because the used solvent system does not separate accurately polar components. The reason for spotting a range of concentration of extract was to determine the minimal amount of fresh tissue, which inhibits microbial growth.

In the case of fungi, a conidial suspension ($5x10^5$ conidia/ml) in FSM[12] medium was sprayed over the developed TLC plate. TLC plates were incubated for 4-5 days in polyethylene boxes (24.3 x 24.3 x 1.8cm Nunc A/S), lined with moistened paper, in the dark at 25 °C. The inhibition of fungal growth indicates the presence of an antifungal compound (Cole 1994).

For testing antibacterial activity the developed TLC plates (20 x 20 cm) were overlaid with 50 ml of nutrient broth containing 0.5 ml glycerol, 1 % agar, 50 mg 2,3,5-triphenyltetrazolium chloride and 10^7cfu of *S. aureus*. The plates were incubated over

[12] FSM Sucrose-salts medium (Cooper and Wood 1975)
Dissolve in dH_2O up to 1l 2g $NaNO_3$, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$ and 10ml trace elements stock solution (Dissolve in dH_2O up to 1l 20mg $FeSO_4.7\ H_2O$, 100mg $ZnSO_4.7\ H_2O$, 2mg $Na_2MoO_4.7\ H_2O$, 2mg $CuSO_4.5\ H_2O$, and 2mg $MnCl_2.4\ H_2O$). Adjust to pH 6.5 before sterilization.

night at 37 °C in polyethylene boxes. The inhibition of bacterial growth was observed as pale spots against a deep pink-red background, due to the conversion of tetrazolium to formazan by active bacteria (Threlfall and Whitehead 1992).

2.4.4 Agar Well Diffusion Assay

Crude extracts from different cultivars, of a volume corresponding to 1 g fresh weight, were loaded into wells cut in the agar medium plate (Malt Agar). EtOH was used as control. Once the crude extract diffused into the agar 50 µl of a 10^6conidia/ml suspension of *T. harziamun* were spread over the agar medium. The plates were incubated at 28 °C for 4 days. Inhibition of fungal growth around the wells indicates the presence of anti-microbial compounds.

2.4.5 Fungal Minimal Active Concentration Determination

Dilutions of reference compounds present in cassava (scopoletin, esculetin, esculin, and rutin) and other reported phytoalexins (naringenin, caffeic acid, kaempferol and phloretin) were loaded onto TLC plates and sprayed with conidial suspensions and incubated as mentioned above. A wide range of concentrations was tested, starting from reported physiological concentrations of these compounds in cassava roots (10, 20, 50, 70, 100, 500nmol and 1, 5, 10, 50, 100 µmol) (Sakai et al. 1994, Buschmann et al. 1998).

2.5 STATISTICAL ANALYSIS.

Data were analysed using ANOVA with PPD level (high, medium and low), cultivars within PPD levels and cultivars as factors. Mean values were separated by REGWQ using the GLM procedure of SAS (SAS Institute, Cary, NC). If *F* values were significant, means were separated by REGWQ test. The ANOVA test was done for each day during the time course for each biochemical measurement (secondary metabolites or enzymatic activity). ANOVA was also performed taking as dependent variable the area under the time course curve for each biochemical measurement. For

this, values of the area under the time course curve (AUTC) were transformed as the ln(AUTC + 1).

A Pearson correlation analysis was also performed to study relationships among the different biochemical measurements, using the CORR procedure of SAS. Two dependent variables were considered, the measurement of each day and the AUTC. Correlations were studied for all cultivars and also among cultivars within PPD levels groups for each of the four groups of samples (see table 2.2).

A Principal component analysis (PCA) was used to determine the main biochemical trait in the PPD response. The PCA was performed only for the December 1999 sample group. PCA was performed using the PRINCOMP procedure of SAS. The first three components of the analysis were selected to construct a three-dimensional graph using the software JMP (version 3.1.4, SAS Institute Inc.).

In order to easy visualise the general trends in secondary metabolites accumulation and enzymatic activity in the different groups of samples (Bath, December 1999, June 1999 and Family K), the average of the cultivars response was calculated dividing the samples in three groups based on the susceptibility of the cultivars towards PPD (low, medium and high). Additionally, the day of maximum accumulation of metabolites and enzyme activity between the three susceptibilities to PPD was determined. It was carried out by the calculation of the peak frequency, it means, the day of the PPD time course at which the highest value was observed. In this case, the quantifications of the three repetitions were not averaged, because the average in the frequency calculation is not statistically representative. This test will be referred to "frequency peak" in the manuscript.

CHAPTER 3

SECONDARY METABOLITES INVOLVED IN PPD

3 SECONDARY METABOLITES INVOLVED IN PPD

3.1 INTRODUCTION

The biochemical changes observed during the progress of PPD are similar to metabolic changes induced by wounding or pathogen attack in other fully studied plant systems. In response to wounding or pathogen attack the phenylpropanoid metabolism is activated.

The phenylpropanoid pathway is one of the most studied biosynthetic pathways in plant natural products. Phenylpropanoids are derived from L-phenylalanine by the action of phenylalanine ammonia lyase (PAL). The phenylpropanoid metabolism can be developmentally regulated and also induced or activated by diverse biotic and abiotic stresses, such as high light or UV light irradiation, wounding, pathogen attack and by low availability of nutrients like nitrogen, phosphorous and iron (Dixon and Paiva 1995).

As reviewed by Solecka (1997), the phenylpropanoid compounds induced in response to wounding or to feeding by herbivores play multiple functions. High levels of coumarin and coumesterol are toxic to herbivores, causing estrogenic and anticoagulant effects. Psoralens can produce photo-induced blistering. Flavonoids and especially tannins, can act as astringents, feeding deterrents, protecting plants from overgrazing by diverse animal species. Chlorogenic acid, ferulate alkyl esters and cell bound phenolics may play roles directly as defence compounds or serve as precursors for the biosynthesis of lignin and suberin. Plant phenolics are multifunctional and can act as reducing agents, free radical terminators, metal chelators and single oxygen quenchers. Phenolic antioxidants also can retard the lipids oxidation by inhibiting lipoxygenase activity, e.g. (-)-epicatechin gallate, (-)-epigallocatechin gallate and epigallocatechin (Amiot et al. 1997).

The products of the phenylpropanoid pathway, which have been more related with the PPD response, are hydroxycoumarins and flavonoids.

Hydroxycoumarins are lactones derived from 2-hydroxycinnamate to give coumarin or from 2-hydroxylated hydroxycinnamates to give hydroxycoumarins. They are accumulated in large amounts by some members of the Rutaceae, Solanaceae and Apiaceae (Strack 1997). Precursor feeding studies demonstrated that the biosynthesis of coumarins proceeds from L-phenylalanine via trans-cinnammic acid and trans-4-coumaric acid (Petersen et al. 1999).

Scopoletin (6-methoxy 7-hydroxy coumarin), one of the hydroxycoumarins found in cassava tissues, has been associated with general defence reactions in plants. The accumulation of this compound in plant tissues, at higher concentration that physiological levels, causes severe stunting, epinasty and foliage yellowing, which render hosts cells less attractive as substrate for pathogens (Sequeira and Kelman as cited by (Goodman et al. 1967). One example of the antifungal activity of scopoletin is the spores' germination inhibition of *Ophiostoma ulmi* the causal agent of the Dutch elm disease (Valle et al. 1997). Scopoletin has also shown to have an inhibitor effect on the oxidation of various substrates by mitochondria but the real mechanism of action stills need to be elucidated, since the inhibition of electron transfer via the respiratory chain of mitochondria was not clear as it was observed for other secondary metabolites as quercetin (Medentsev and Akimenko 1999). Other studies on scopoletin activity have suggested that this metabolite may induce testicular failure at the level of sperm maintenance in guinea pigs (Obidoa et al. 1999).

Flavonoids, in the general sense (e.g. anthocyanins, isoflavonoids, proanthocyanins, catechins and condensed tannins), constitute the second group of secondary metabolites derived from phenolic metabolism, which have been related with PPD. These secondary metabolites are formed by two different secondary pathways: the phenyl propanoid pathway and the polyketide pathway (Petersen et al. 1999). The key enzyme for flavonoid biosynthesis is chalcone synthase (CHS). Flavan-3,4-diols (leucoanthocyanidins) are reduced to flavan-2,3-trans-3-ols by a NADPH-dependent leucoanthocyanidin 4-reductase (LAR). This enzyme transforms leucocyanidin to (+)-catechin and leucodelphinidin to (+)-gallocatechin (Petersen et al. 1999).

Flavonoids play many different roles like pigments and UV protectants. In legumes, flavonoid derivates also play key roles in the interaction with microorganisms. Hence, symbiotic nitrogen fixing *Rhizobia* and *Bradyrhizobia* recognise flavones, flavanones, chalcone and isoflavones released to the rhizosphere as signals for the activation of their nodulation genes, while isoflavonoids are the major structural class of phytoalexins in legumes (Dixon et al. 1994).

Flavonoids are end products and may remain unchanged all over the lifetime of a plant. Nevertheless, turnover and degradation may occur. Oxidation reactions, catalysed by peroxidases, can lead to polymerisation, which is evidenced as browning substances in injured tissues (Strack 1997).

Flavan-3-ols derive from flavan-3,4-cis-diol mediated by NADPH-dependent flavan-3,4-diol 4-reductase, which is involved in the biosynthesis of catechin and its relatives. Flavan-3-ols are important structural elements of condensed tannins (Strack 1997).

The polymerisation of phenolic metabolites leads to the production of lignins and tannins.

Lignins are complex phenyl propanoid polymers mainly found in the secondary walls and middle lamellae of cell walls of tracheids, vessels, fibres, etc., in vascular plants (Lewis and Yamamoto 1990). They are formed by polymerisation of monolignols (hydroxycinnamyl alcohols: 4-coumaryl, coniferyl and sinapyl alcohols) in a reaction mediated by peroxidases in presence of H_2O_2. Lignin deposition starts towards the end of primary cell growth at the cell corners and the middle lamella. The initial steps might be attachments (ester bonds) of hydroxycinnamates to specific sites in the cell wall polysaccharides. Then, coupling of these hydroxycinnamates catalysed by peroxidases form the associations between the growing lignin polymers and the polysaccharides (Strack 1997).

Lignin can act as a physical barrier, preventing enzymes from pathogens penetrating the tissues, and as a chemical barrier as it binds with the cell wall components, by covalent linkages with hemicellulose or uronic acid ester linkages with the non cellulose carbohydrates (Vance et al. 1980)

Tannins are water-soluble plant polyphenols, which cause protein precipitation from aqueous solutions. They are oligo- and polymeric phenolics, which are divided in two classes: hydrolysable and condensed tannins. In contrast to lignin, both tannin classes are located in vacuoles (Haslam 1981). The importance of tannins lies in their effectiveness as repellants to predators, animal or microbial. The high astringency of tannins renders the tissues unpalatable by the precipitation of salivary proteins in animals. In parasitic organisms, tannins delay the invasion of plant tissue by immobilizing extracellular enzymes (Haslam 1981).

PPD can be observed as an oxidative process. Therefore, it is of particular interest the presence of metabolites with antioxidant activity in cassava roots. Antioxidants have been classified in two groups: primary or chain breaking antioxidants which react with lipid radicals to transform them in more stable products and secondary or preventive antioxidants which reduce the rate of chain initiation or decompose hydroperoxides to non radical species (Amiot et al. 1997). The activity of catechins, gallocatechins, catechin gallate esters and other phytochemicals has been studied and has been

undertaken in models of cancer diseases and markers for lipid metabolism. These metabolites have been proven *in vitro* assays to be more active antioxidants than vitamin C (reviewed by Rice-Evans et al. 1997).

Other antioxidant nutrients are present in cassava roots such as ascorbic acid and β-carotene (Chavez et al. 2000).

In this chapter, secondary metabolites derived from the phenylpropanoid pathway will be detected and quantified in cassava roots undergoing PPD. The study of the pattern of accumulation of these metabolites will contribute to a better understanding of the physiological changes occurring during post-harvest deterioration.

3.2 RESULTS

3.2.1 Management of samples

Experiments were conducted on four groups of samples. The first group was composed of cassava roots harvested at CIAT and processed at Bath. This group will be referred throughout the manuscript as Bath sample group. Since these roots were not processed immediately after harvest, the deterioration time course during which experiments were performed will be called storage time course and not post-harvest time course. Cassava cultivars evaluated in this first group were: CM 2177-2, MCOL 22, CM 7033-3, SM 985-9, MDOM 5, MNGA 2, MNGA 1 and MBRA 337.

The second and third group of samples consisted of roots, harvested and processed at CIAT in June 1999 and December 1999 respectively. Cassava cultivars in the second group were: CM 2177-2, MCOL 22, MNGA 2, MVEN 77, MBRA 12 and MPER 183. The third group of samples comprised cultivars: CM 2177-2, MCOL 22, CM 7033-3, SM 985-9, MDOM 5, MNGA 2, MVEN 77, MBRA 12, MPER 183 and MBRA 337.

The difference between the second and the third group of samples was that in June 1999, each sample consisted of one entire root and in December 1999, each sample was only a portion of the root (see chapter 2.1.3). These two groups of samples will be referred as June 1999 and December 1999 samples.

The fourth group of samples was harvested and processed at CIAT and is called Family K because it is composed of samples from 35 genotypes of the mapping population.

In order to characterize the biochemical events occurring during post-harvest deterioration, cassava cultivars were classified in three groups according to their response to PPD (low, medium and high PPD levels). Then, comparisons and statistical analyses (ANOVA, REGWQ mean separation groupings) were made for the biochemical measurements between the groups as well as among all cultivars.

The classification of the sampled Family K members as low, medium and high PPD cultivars did not follow the same pattern as other cultivars. As it was mentioned in chapter two, cultivars with scores of PPD between 0 and 20 % were grouped as low susceptible cultivars, cultivars with PPD scores between 21 and 40 % are classified as medium susceptible cultivars and cultivars between 41 and 100 % are grouped as high susceptible cultivars. The family K members were selected for their response to PPD in an evaluation performed in December 1998. Details on the selective criteria for the family K members genotypes are given in section 5.2. The difficulty in the PPD level classification for the family was based on the variation for the scores between December 1998 and December 1999. In general PPD scores during December 1999 harvesting were lower than the scores for December 1998. The frequencies of the results of PPD visual scores for the entire family K population in the two harvesting seasons will be presented in chapter five. In order to determine the PPD susceptibility of the 35 family K genotypes, the scores for 1998 and 1999 were plotted in a two dimensional graph (fig. 3.1). Then, a diagonal line was drawn across the graph to ease the observation of the PPD score differences between the two seasons. If the PPD scores for both years had been similar, all points in the graph should be around the perpendicular line. After that, squares marking the values for low, medium and high PPD levels were drawn over the graph. Finally, the genotypes located in the green square (0-20 % PPD) were classified as low PPD level, the genotypes in the orange squares (21-40 % PPD) were classified as medium PPD level, and the genotypes in the purple square (41-100 % PPD) were classified as high PPD level.

The graphs presented in this chapter and chapter four represent the mean of the values obtained for the different secondary metabolites and enzyme assays quantifications, from the three replicas. The error bars correspond to the standard deviation based on the entire population.

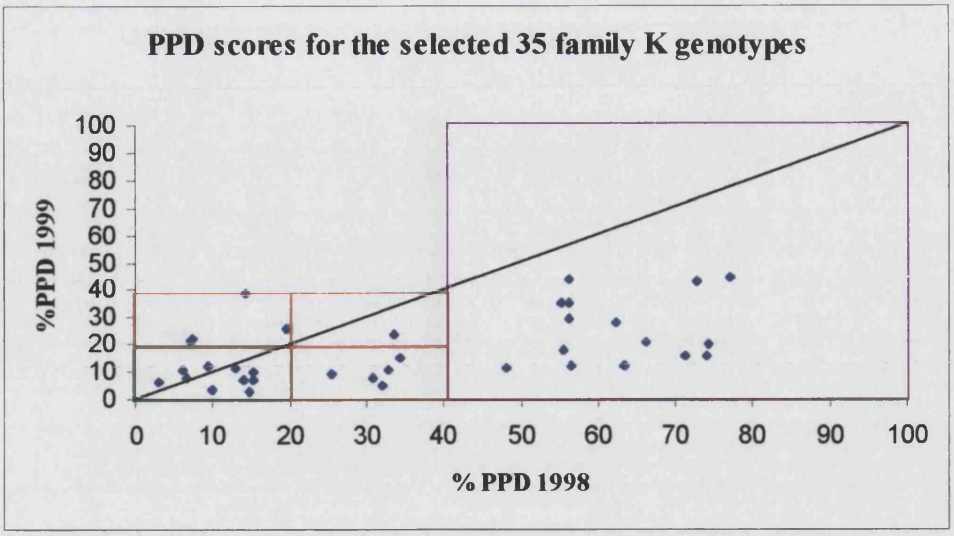


Figure 3.1 PPD scores for 35 genotypes of the family K al CIAT-Palmira during 1998 and 1999.

3.2.2 Observations on deterioration of cassava roots.

Observations in day- and UV-light (253 nm and 366 nm) confirmed the induction of vascular streaking symptoms under the conditions used for the roots processed at Bath (Chapter 2.1.2) as it occurs in complete roots after harvest. Depending on the cassava cultivar, browning of the vascular tissue was observed after two to four days of storage. Observation of the root slices under UV-light over the whole storage period showed an increase of blue fluorescence in the storage parenchyma during the first three to six days (depending on the cultivar). After that period, with the beginning of fungal growth on the root slices, this fluorescence decreased or was overlaid by other colour effects.

Microscopic observations showed the occlusion of some of the xylem vessels as well as the parenchymatic cells by brown compounds (Fig. 3.2). In addition, occlusions by tyloses into big xylem vessels could be observed.

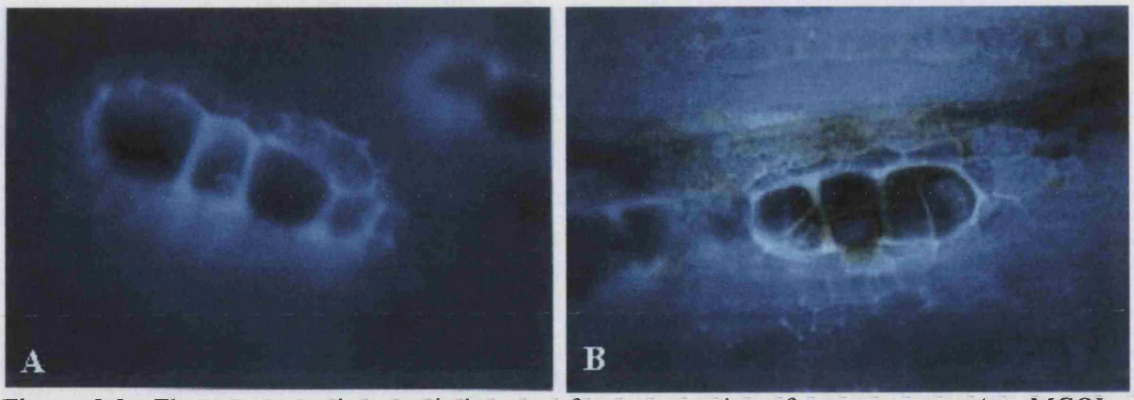


Figure 3.2. Fluorescence microscopic images of a cross section of a cassava root (cv. MCOL 22). A) Root cross section after one day of storage (x400). B) Root cross section after four days of storage (x400).

The PPD response of the roots processed at Bath showed a high variability in individual roots and did not depend on the site of growth (Bath greenhouse or CIAT). This variability within cultivars was noticed as well in the roots grown and processed at CIAT. For example cultivar MCOL 22, which was considered a high PPD response variety, showed different susceptibilities during this study. MCOL 22 behaved as a high PPD variety in the group of samples processed at Bath and CIAT during June 1999, but with the group of samples collected at CIAT during December 1999 its response was medium. Another case was cultivar MDOM 5, which was considered for several years as low PPD, and was used as a good low PPD candidate for breeding studies (C. Iglesias, pers. comm.). This cultivar showed one of the higher PPD responses amongst the cultivars used in this research. CIAT researchers have also observed this high variability of the PPD response within cultivars and harvesting seasons for several years.

Figure 3.3a shows the occurrence of vascular streaking (transverse cut root slices) in different cultivars over a post harvest time course of four days. The earlier discoloration of the vascular parenchyma in susceptible cultivars was very clear compared to low susceptible ones. The progress of vascular streaking from the proximal root end along the whole root at three days after harvesting is shown in figure 3.3b. As in figure 3.3a, as well the high severity of vascular streaking in the susceptible cultivars was obvious.

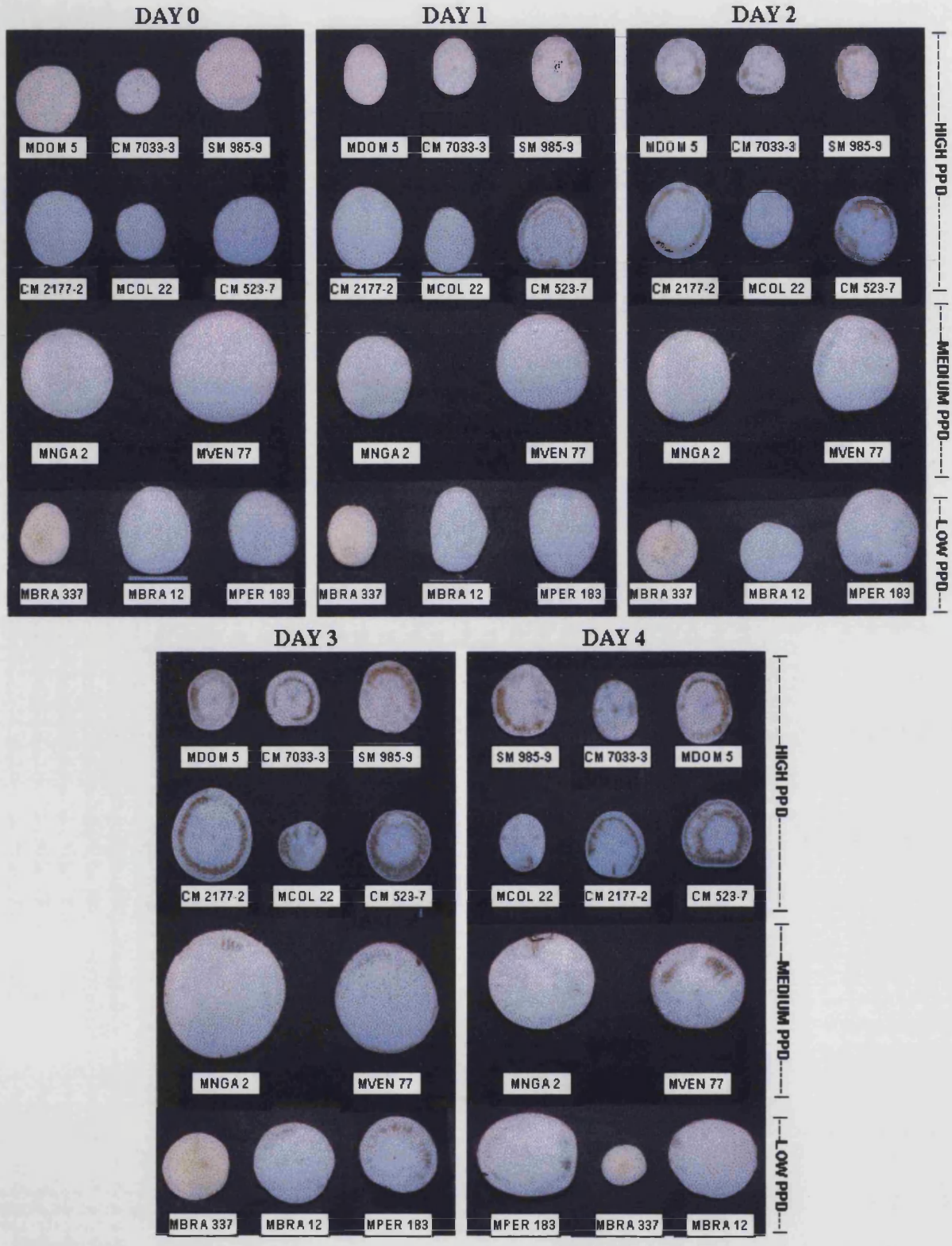


Figure 3.3a Vascular streaking symptoms in cultivars with different responses towards PPD during a post harvest time course of four days. High PPD cultivars: MDOM 5, CM 7033-7, SM 985-9, CM 2177-2, MCOL 22 and CM 523-7. Medium PPD cultivars: MNGA 2 and MVEN77. Low PPD cultivars: MBRA 337, MBRA12 and MPER 183.

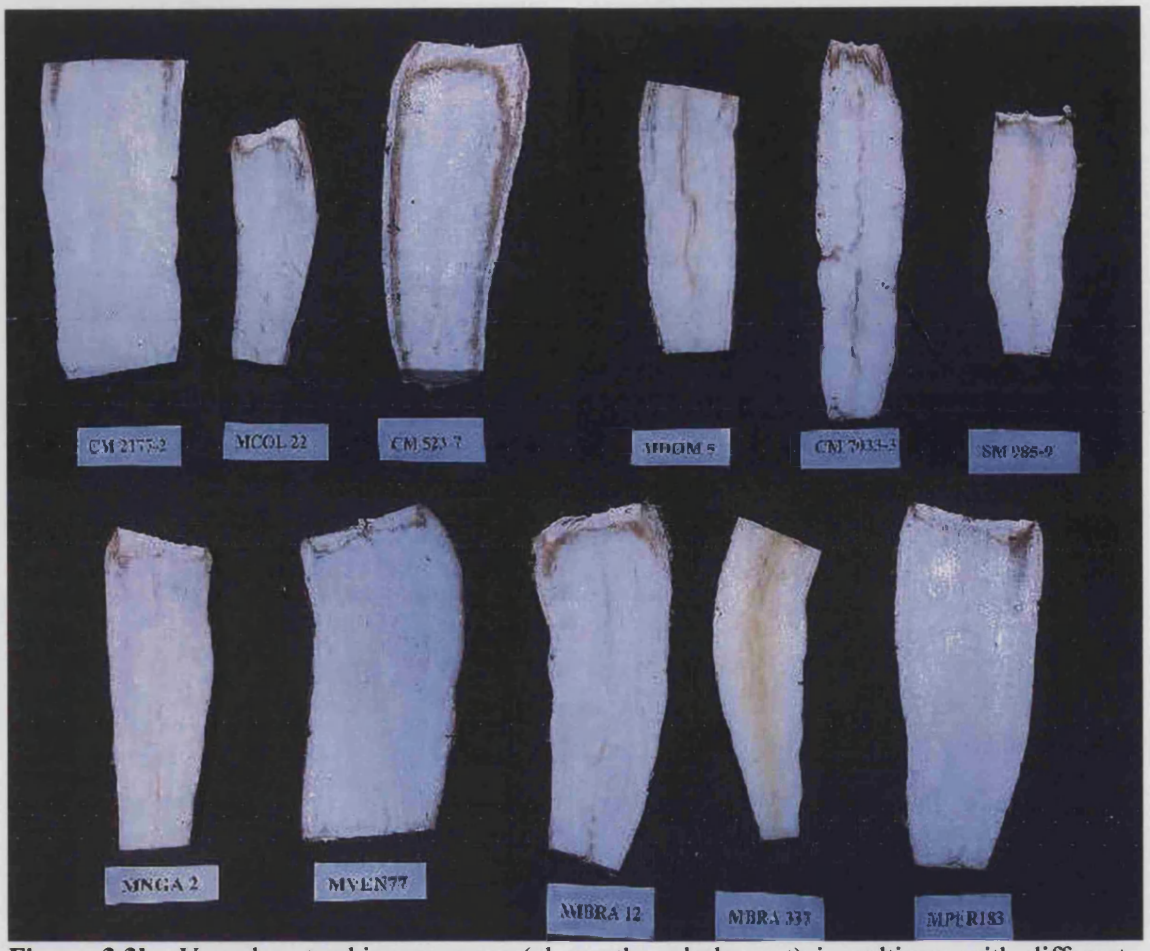


Figure 3.3b Vascular streaking progress (along the whole root) in cultivars with different responses towards PPD, at three days after harvest. High PPD cultivars: MDOM 5, CM 7033-7, SM 985-9, CM 2177-2, MCOL 22 and CM 523-7. Medium PPD cultivars: MNGA 2 and MVEN 77. Low PPD cultivars: MBRA 337, MBRA12 and MPER 183

3.2.3 Determination of the soluble phenol content in ethanolic root extracts

The quantification of the total phenol content in cassava root tissue undergoing PPD can be used as a sign of the phenylpropanoid pathway activation. Initial experiments to determine the total phenol content in ethanolic root extracts gave no clear results because of high fluctuations of the content. These fluctuations could be the caused by different carotene contents in the extracts. Carotenes are poorly soluble in ethanol and may dissolve in different concentrations during the extraction process of the samples. Carotenes cause a photometric reaction with the Folin-Ciocalteau reagent overlaying the reaction with the other phenolics in the extracts leading to significant misinterpretations. This is the reason why the protocol proposed by Cliffe et al. (1994) was modified using the extract diluted 100 fold in dH_2O. Dilution of the extract in water, followed by cooling at 4 °C for 1 hour and centrifugation, before the colour reaction, helps to remove the carotenes and the total phenol content can then be measured from the

supernatant without interference.

The soluble phenol content of ethanolic extracts was measured in the groups of samples processed at CIAT. No clear tendency was observed in the accumulation of the soluble phenolics. Figure 3.4 shows the soluble phenol content expressed in gallic acid equivalents (GAE) of three cultivars, CM 2177-2, MNGA 2 and MBRA 12, with contrasting responses to PPD, high, medium and low respectively. There was not a marked tendency in the accumulation of the soluble phenol content. Only the high susceptible cultivar showed an increase of concentration after five days of harvesting.

The soluble phenolics concentration graphs in appendix 8.4.2 showed higher accumulation in the general time course for the high PPD cultivars in June 1999 group data, but the December 1999 group data showed the lowest accumulation of soluble phenolics for the high PPD cultivars. Looking at these results, it was not possible to determine a direct association between the accumulation of soluble phenolics and susceptibility to PPD. This observation was also confirmed by the results of the ANOVA analysis (REGWQ groups, appendix 8.3.1), where no significant differences between susceptibility levels were found along the post harvest storage time course. Neither was it possible to detect a general tendency in accumulation of soluble phenolics since no marked peaks of concentration were observed (see appendix 8.4). Only the Family K data group presented a marked peak in accumulation on the fourth day after storage, which was common for the different levels of PPD susceptibility (appendix 8.4.4).

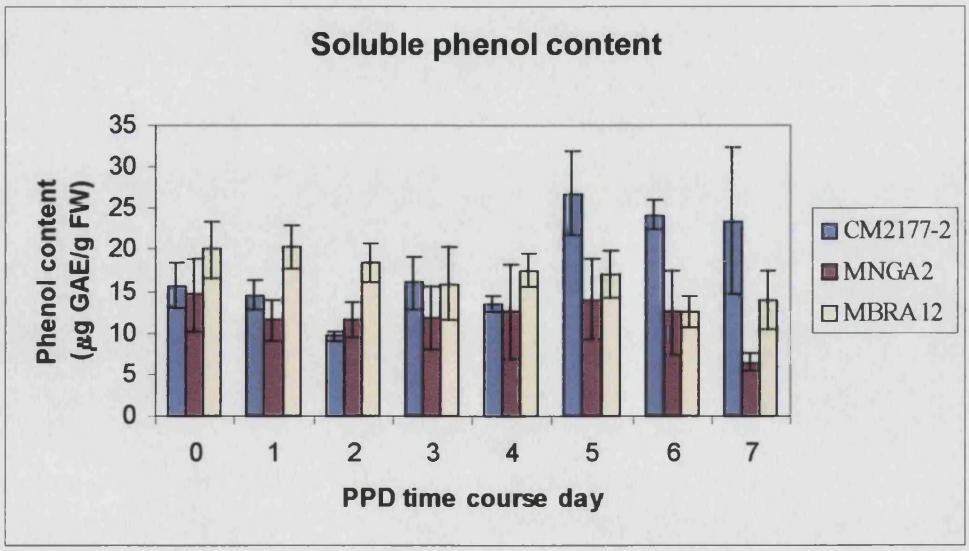


Figure 3.4. Soluble phenol content of cassava root ethanolic extracts undergoing PPD, in cultivars with contrasting responses to PPD (high: CM 2177-2, medium: MNGA 2 and low: MBRA 12). Details on represented values, population size and error bars are given on section 3.2.1.

3.2.4 Identification of secondary metabolites associated with PPD

3.2.4.1 Chromatographic Analysis

3.2.4.1.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a relatively simple technique that can help to identify secondary metabolites that are constitutive in cassava or synthesised in response to PPD. TLC patterns of ethanolic root extracts of different cultivars showed no significant differences during the time course of storage. With the HPTLC chromatograms of crude ethanolic extracts of samples processed at Bath it was possible to detect seven compounds by comparison with reference compound retention factors (R_f). The compounds are scopoletin (R_f 0.56), (+)-catechin and (-)-epicatechin (R_f 0.41), (-)-epicatechin gallate (R_f 0.36), scopolin (R_f 0.17), esculin (R_f 0.14) and rutin (R_f 0.04). The bands corresponding to scopoletin, scopolin and esculin presented strong blue fluorescence under UV light characteristic of coumarin metabolites (Fig. 3.5).

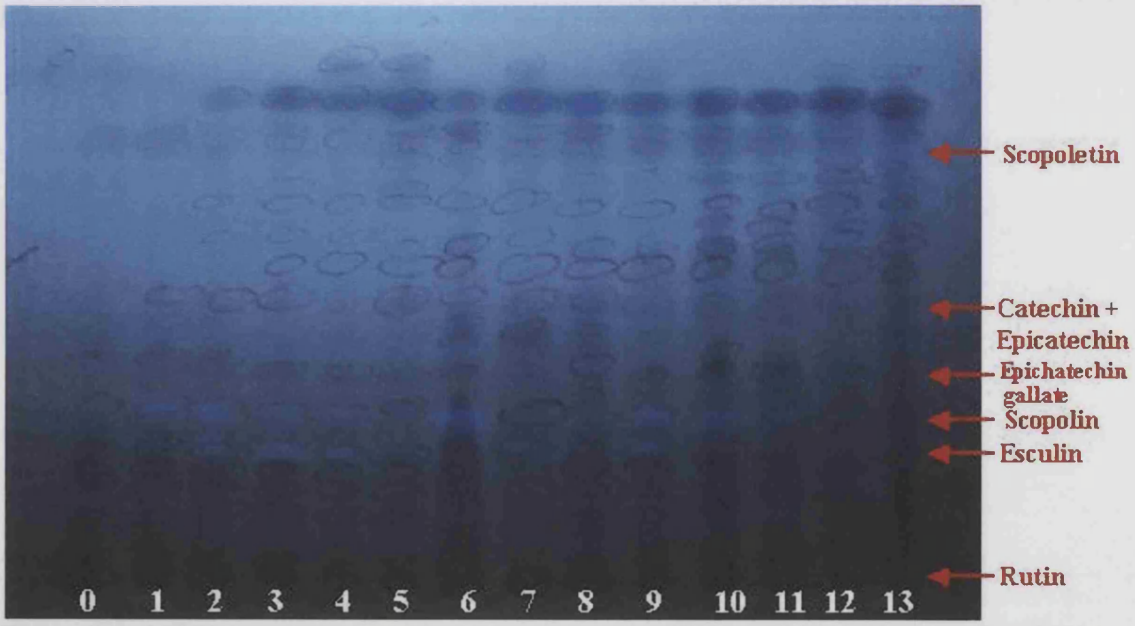


Figure 3.5. HPTLC pattern of cassava root extracts (cv. MBRA 337), observed under UV_{366nm}, during a time course of 13 days of storage.

In order to obtain information of the chemical nature of the metabolites present in cassava root extracts, the HPTLC plates were stained with different reagents. Diphenylboric acid-2- aminoethyl ester reagent or Neu's reagent was used to detect flavonoids and coumarins. The chromatographic patterns of June 1999 group samples

are summarised in appendix 8.1.1. Figure 3.6 shows a HPTLC pattern of cultivar CM2177-2 during a post harvest time course of seven days stained with Neu's reagent and visualised under long UV light. Bands marked with blue arrows show positive coloration for flavonoids. Most of those bands started to accumulate after four to five days after storage. The coumarins, scopoletin and scopolin show the characteristic blue fluorescence.

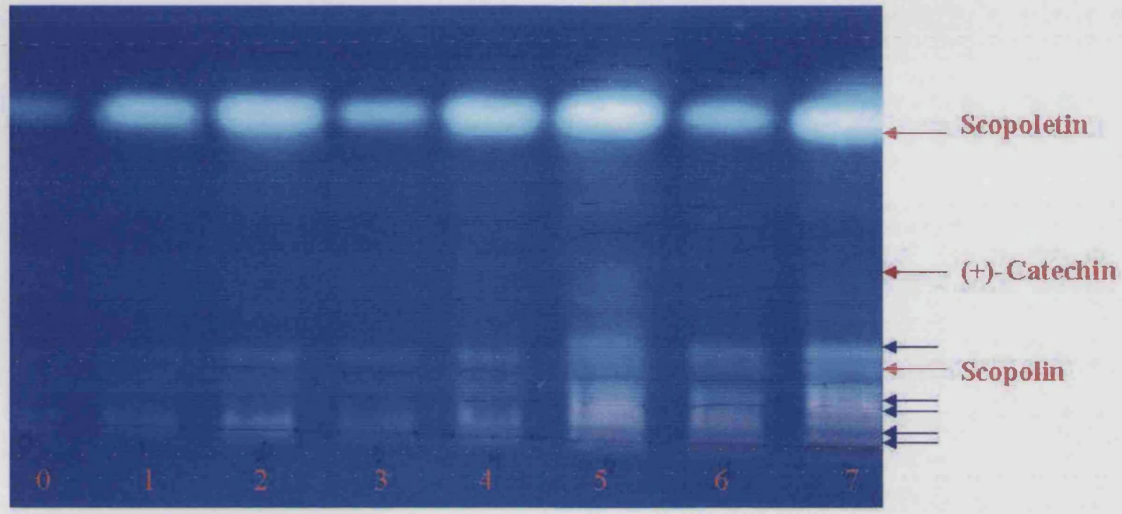


Figure 3.6. Detection of flavonoid compounds in cassava root ethanolic extracts. HPTLC pattern of cultivar CM 2177-2 during post harvest time course of seven days. The HPTLC plate was stained with Neu's reagent and visualised under UV_{366nm}.

HPTLC plates were also stained with antimonium III chloride in order to detect the presence of terpenoid compounds in the root ethanolic extracts. HPTLC patterns for terpenoids are shown in figure 3.7. Bands marked with red arrows show the presence of terpenoid like compounds. Some of them started to accumulate by one or two days after harvesting while others were present before harvesting. Terpenoids have been reported as stress metabolites in cassava (Sakai et al. 1986), but reference compounds were not available to use as references because they were not commercially available. Thus, it was not possible to determine if any of the terpenoid-like bands corresponded to the terpenoids previously found in stressed cassava plants.

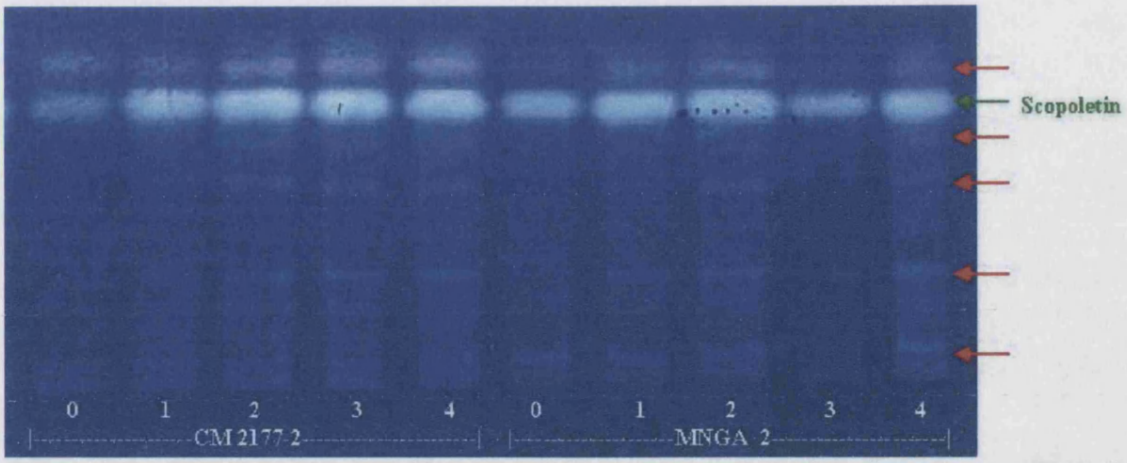


Figure 3.7. Detection of terpenoid compounds in cassava root ethanolic extracts. HPTLC pattern of cultivars CM 2177-2 and MNGA 2 during a post harvest time course of four days. The HPTLC plate is stained with antimonium III chloride and visualised under UV_{366nm}.

The antioxidant characteristic of metabolites present in the root ethanolic extracts was determined by spraying HPTLC plates with 1,1-diphenyl-2-picryl-hydrazyl reagent (DPPH). The patterns of antioxidant bands for samples processed in June 1999 are summarised in appendix 8.1.2. In all samples a smear was observed from the base line to approximately one third of the area delimitated by the solvent front (Fig 3.8). This smear did not allow the determination of the presence of antioxidant bands at the beginning of the chromatogram. Most of these bands were present during the whole PPD time course.

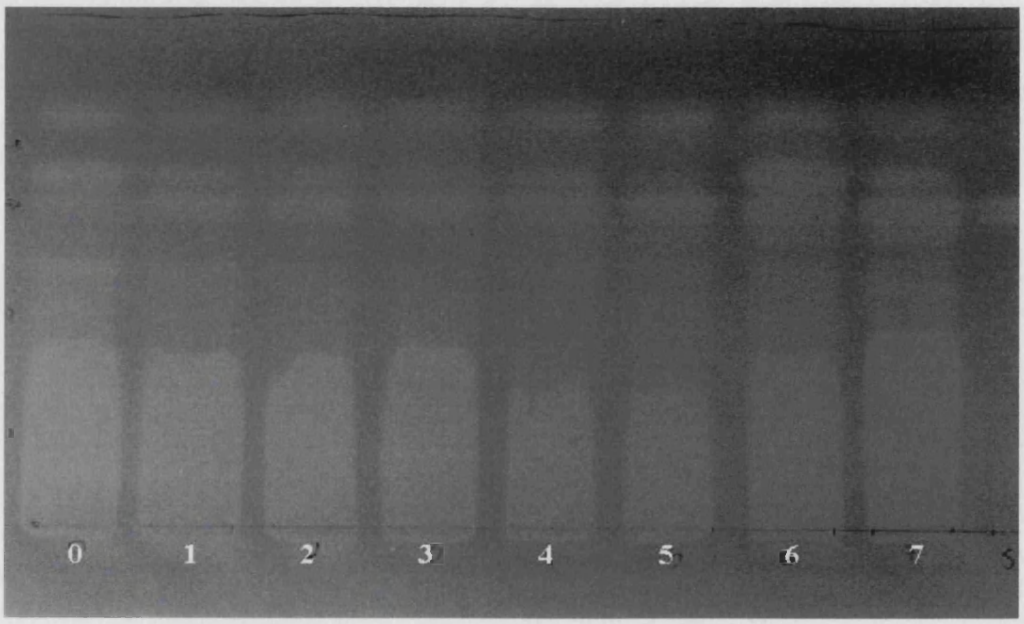


Figure 3.8. Detection of the antioxidant activity of metabolites present in cassava root ethanolic extracts. HPTLC pattern of cultivar MVEN 77 during a post harvest time course of seven days. The HPTLC plate was stained with the DPPH radical and visualised under daylight.

3.2.4.1.2 TLC markers for PPD

Most of the bands detected in the HPTLC plates were present in all extracts or occurred after four to five days after wound stress. In HPTLCs of samples processed at Bath two bands, R_f 0.19 and R_f 0.66, occurred after two to four days of storage and their intensity increased during the storage time (Fig. 3.9). The occurrence and intensity of these bands seemed to be related with the onset of the PPD response of each cultivar. For example in MCOL 22 the intensity of the R_f 0.66 band during days two to four was stronger than MBRA 337. These bands were also present in extracts of complete roots (from the greenhouse) stored during the same period of time. This indicates that these bands were not artefacts produced by slicing.

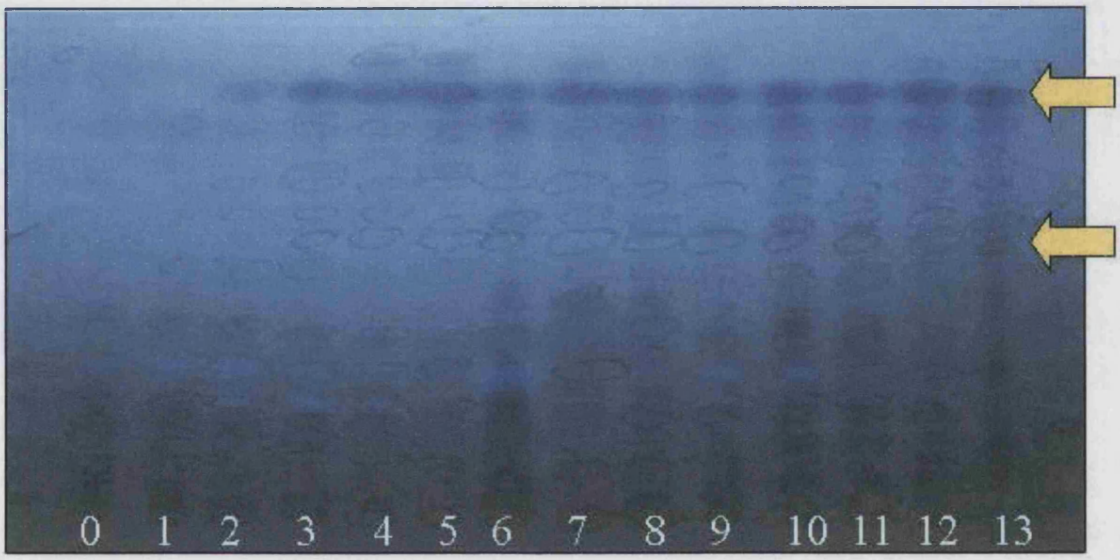


Figure 3.9. Detection of possible marker bands for PPD. HPTLC pattern of cultivar MBRA 337 during a time storage time of 13 days. The band patterns were visualised under UV_{366nm}. Yellow arrows indicate the bands possibly related with PPD.

3.2.4.1.3 High Performance Liquid Chromatography

HPLC chromatograms of crude ethanolic root extracts showed significant differences during the time course of storage (Fig. 3.10).

The comparison of retention times and UV spectra of reference compounds and HPLC patterns of ethanolic root extracts lead to the identification of nine compounds. Four coumarins (esculin, esculetin, scopolin and scopoletin), four catechins ((+)-catechin, (-)-epicatechin, (+)-gallocatechin and (+)-catechin gallate and one flavonoid (rutin) were identified. Figure 3.11 shows some of the identified secondary metabolites in a HPLC run of MBRA 337 cultivar.

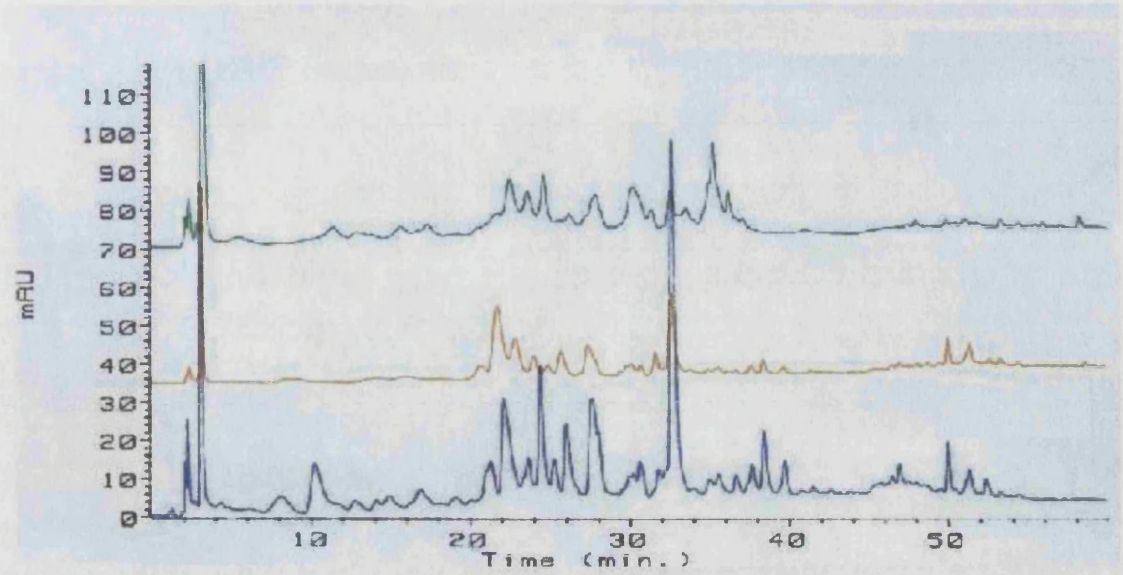


Figure 3.10. HPLC profiles of cassava root ethanolic extracts undergoing PPD. Chromatographic patterns of cultivar MBRA 337 immediately after slicing (green line), after four days (orange line) and after eight days storage (blue line).

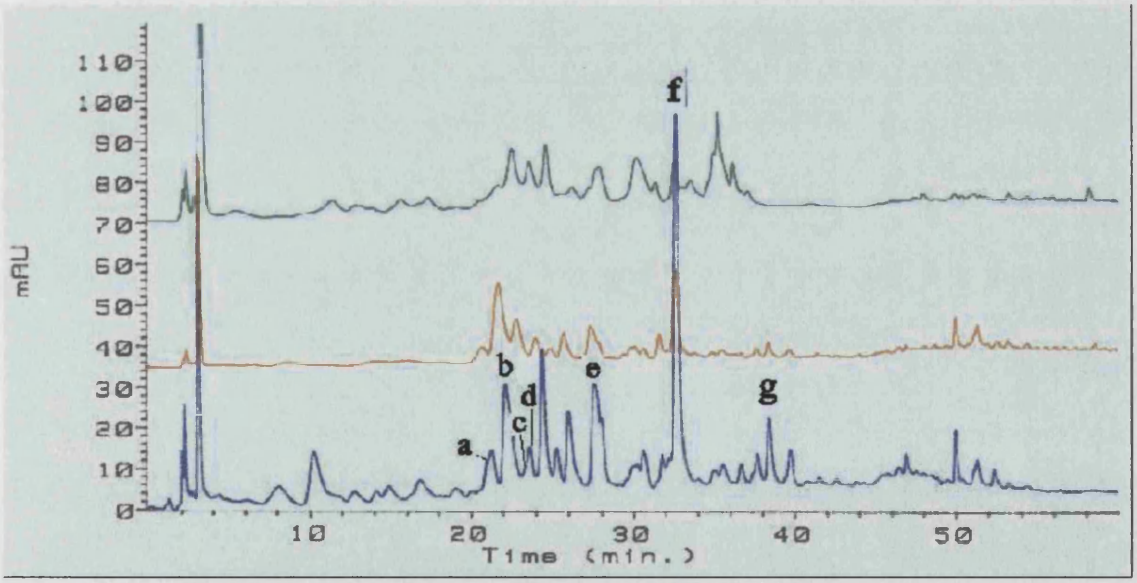


Figure 3.11. Secondary metabolites identification by HPLC. Chromatographic patterns of cultivar MBRA 337 immediately after slicing (green line), after four days (orange line) and after eight days storage (blue line). Identified compounds are: a) esculin, b) (+)-catechin, c) scopolin, d) esculetin, e) (-)-epicatechin, f) scopoletin and g) rutin.

Table 3.1 summarises the retention times and UV maxima wave lengths of reference compounds and peaks identified in MBRA 337 ethanolic extracts. Figure 3.12 shows some of the UV spectra (measured by the diode array detector) of references and identified peaks in cultivar MBRA 337.

	MBRA 337		REFERENCE		
COMPOUND	**RETENTION TIME (min)**	**UV max ABS (nm)**	**RETENTION TIME (min)**	**UV max ABS (nm)**	**REFERENCE**
Esculin	22.2	200, 224, 244, 292, 330	21.6	223, 248, 297, 335	Dubois et al, 1990
(+)-Catechin	23.3	202, 226, 276	23.3	280	Guyot et al, 1996
Scopolin	24.6	202, 226, 246, 254, 288, 336	23.2	205, 227, 289, 340	Kuroyanagi et al, 1986
Esculetin	25.1	200, 226, 252, 288, 338	25.9	230, 250, 260, 344	Razdan et al, 1987
(-)-Epicatechin	28.1	200, 272	27.8	231, 280	Bradfield and Penney, 1948 Giner et al, 1993
Scopoletin	32.5	202, 226, 256, 294, 342	32.2	225, 240, 245, 346	Razdan et al, 1987
(+)-Gallocatechin	33.9	200, 275	33.6	224, 271	Bradfield and Penney, 1948
Rutin	35.9	210, 256, 262, 294, 356	35.8	256, 268, 296, 362	Ulubelen et al, 1980

Table 3.1. Summary of HPLC data of identified peaks in cultivar. MBRA 337 ethanolic root extracts and compound references.

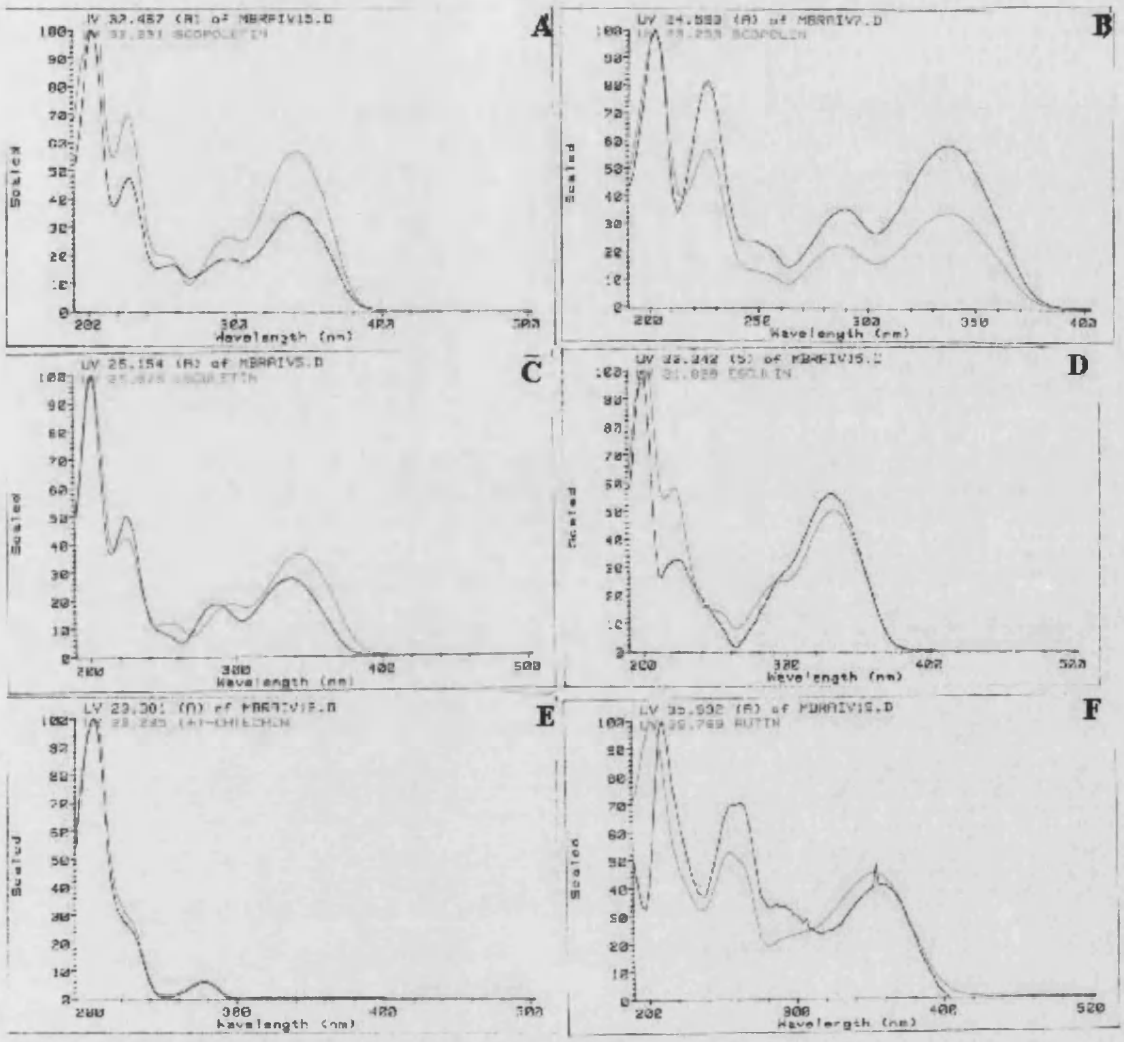


Figure 3.12. Comparison of UV spectra (190-400nm) of reference compounds (orange line) and HPLC peaks (blue line) with similar retention time. A) scopoletin, B) scopolin, C) esculetin, D) esculin, E) (+)-catechin and F) rutin.

3.2.4.1.4 Compound Patterns and Markers for Post Harvest Physiological Deterioration

In all cultivars the occurrence of new peaks after 50 minutes retention time was observed (Fig. 3.13). The height and appearance time (two to four days) of these peaks may suggest a relation with the PPD susceptibility of the different cultivars.

According to the results obtained with HPTLC, the bands related with PPD (R_f 0.19 and 0.66) were isolated from the plates and injected in the HPLC. It was found that the "PPD band" (R_f 0.66) had a retention time of 50 minutes and its UV spectrum was identical to the UV spectrum of one of the highest "PPD related" HPLC peaks (Fig. 3.14).

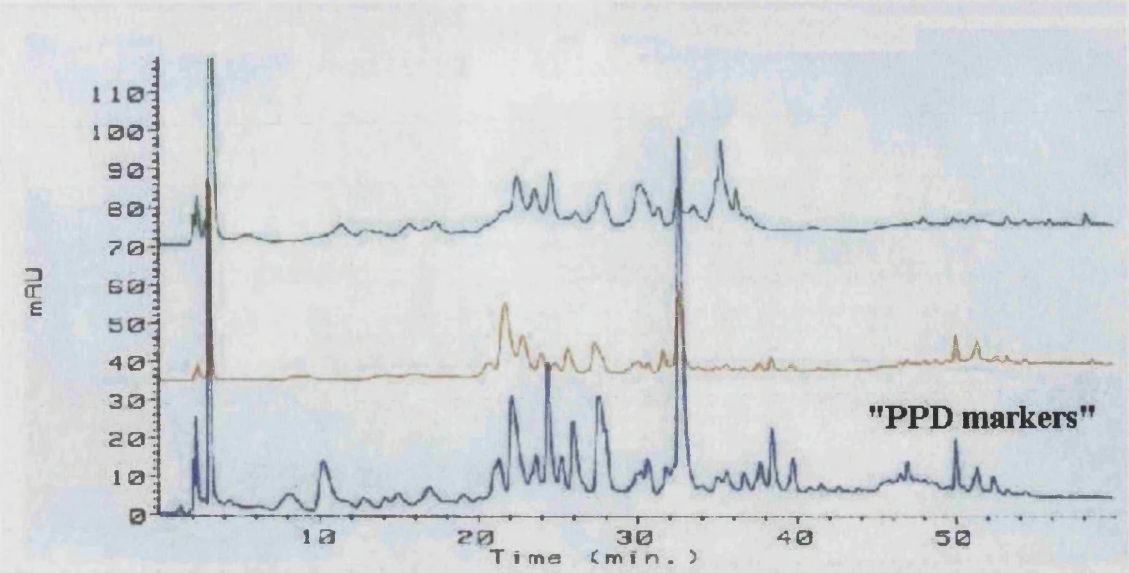


Figure 3.13. HPLC profiles of cassava root ethanolic extracts undergoing PPD showing the possible marker compounds for PPD. Chromatographic patterns of cultivar MBRA 337 immediately after slicing (green line), after four days (orange line) and after eight days storage (blue line).

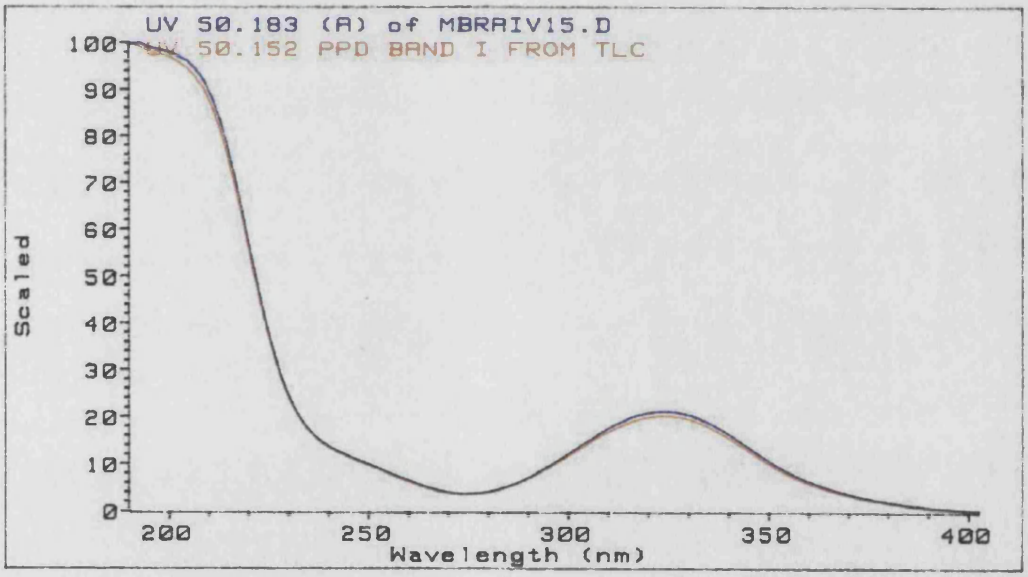


Figure 3.14. Comparison of UV spectra (190-400nm) of "PPD related band" isolated from HPTLC plates (orange line) and "PPD related HPLC peak" (blue line).

3.2.4.2 Confirmation of secondary metabolites identity by spectroscopic analysis

The analysis of cassava root ethanolic extracts by spectroscopic methods (UV and MS) combined with LC confirmed the detection of four hydroxycoumarins (scopoletin and esculetin and their respective glycones scopolin and esculin) and three flavan-3-ols ((+)-catechin, (+)-gallocatechin and (+)-catechin gallate). The spectroscopy data of those metabolites is given in table 3.2 and some UV spectroscopy profiles are shown in figure 3.15. The structures for the hydroxycoumarins and flavan-3-ols and flavonoid metabolites determined are given in figures 3.16.and 3.17 respectively.

Compound	**Molecular Formula**	**Mass $[M+H]^+$ (rel. intensity)**	**Fragments (rel. intensity)**	**UV_{max} (50% CH_3CN)**
Esculin	$C_{15}H_{16}O_9$	341 (55)	227 (100), 179 (18), 147 (10)	224, 244, 292, 330
Esculetin	$C_9H_6O_4$	179 (70)	163 (100), 131 (45)	226, 252, 288, 388
Scopolin	$C_{16}H_{18}O_9$	355 (10)	193 (100)	202, 226, 246, 254, 288, 336
Scopoletin	$C_{10}H_8O_4$	193 (100)	163 (5)	202, 226, 256, 294, 342
(+)-Catechin	$C_{15}H_{14}O_6$	291 (100)	272 (20)	202, 226, 276
(+)-Gallocatechin	$C_{15}H_{14}O_7$	306 (100)	139 (20)	204, 228, 269
(+)-Catechin gallate	$C_{22}H_{18}O_{10}$	460 (70)	118 (100)	200, 218, 274

Table 3.2. Mass and UV spectroscopic data of identified hydroxycoumarins and flavan-3-ols of cassava root extracts.

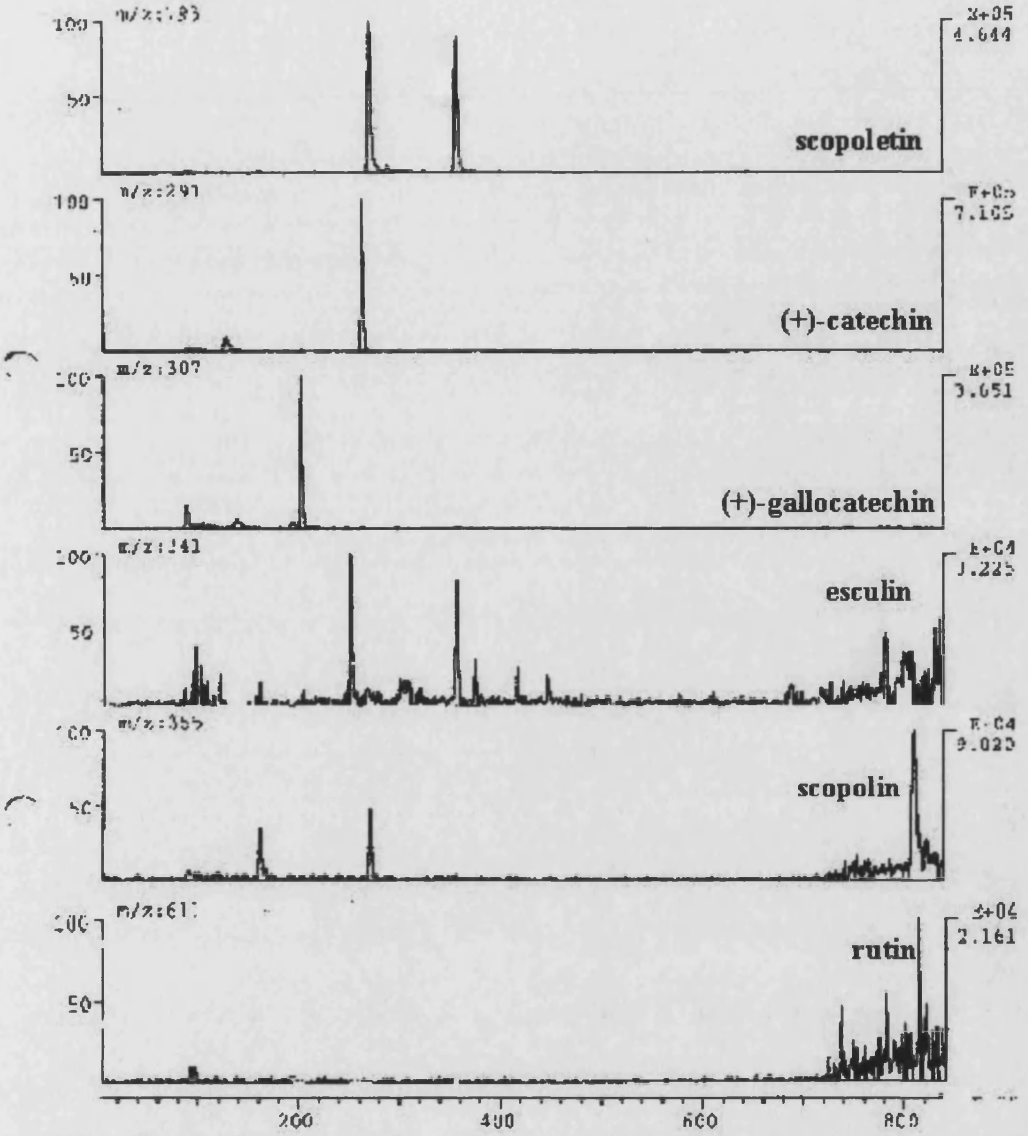
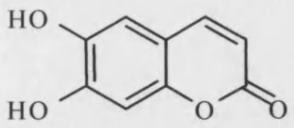
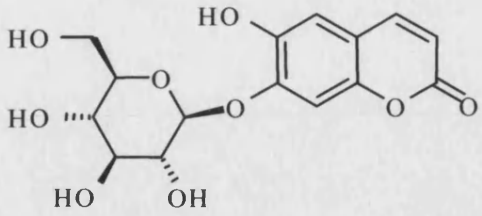


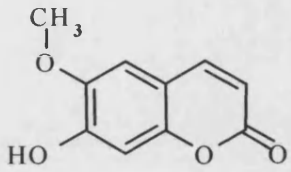
Figure 3.15. UV spectroscopic profiles of some secondary metabolites present in cassava root ethanolic extracts.



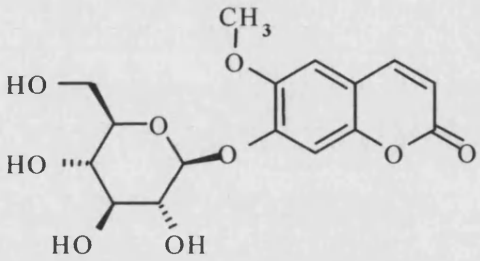
Esculetin



Esculin

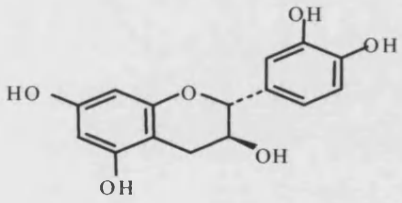


Scopoletin

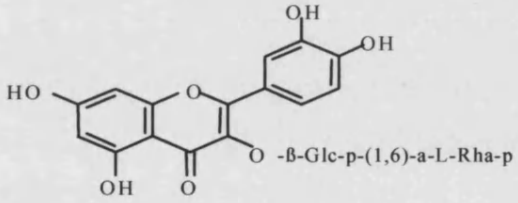


Scopolin

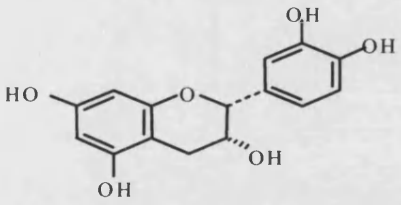
Figure 3.16. Structures of hydroxycoumarins identified in cassava root extracts.



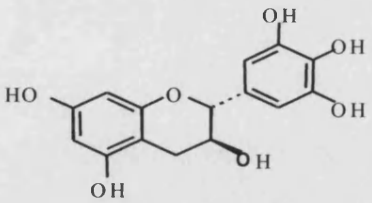
(+)-Catechin



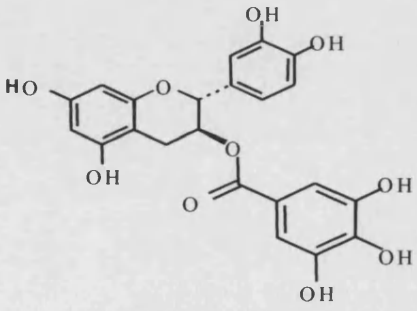
Rutin



(-)-Epicatechin



(+)-Gallocatechin



(+)-Catechin gallate

Figure 3.17. Structures of flavan-3-ols and flavonoid found in cassava root extracts.

3.2.5 Quantitative changes of hydroxycoumarins

The four hydroxycoumarins were detected in all samples processed at Bath, but not in the other sample groups. Esculin and esculetin were not easily detected in the group of samples processed at CIAT during June 1999, and esculetin was not quantifiable in the CIAT's December 1999 and family K groups of samples.

Figure 3.18 shows the hydroxycoumarin accumulation profiles for two PPD contrasting cultivars over a storage time course of seven days. Although high variability between replicates was observed, particular tendencies can be highlighted. Directly after wounding small amounts of the four coumarins were detectable for both cultivars. In MCOL 22 a considerable increase of scopoletin occurred after one day of storage, followed by a large increase of scopolin after the second day. An accumulation of scopoletin after one day in MBRA 337 was observed but not as large as occurred place with MCOL 22. In the susceptible cultivar, the concentration of esculetin and esculin remained very low compared with scopolin and scopoletin between days two and four. Scopolin and scopoletin decreased after five and six days respectively, which was opposite to the MBRA 337 accumulation profile. The highest concentrations of scopolin and scopoletin were detected after the sixth day for MBRA 337. Another difference between both cultivars was that amounts of esculetin and esculin were higher in MBRA 337, and it was possible to observe peaks at four and five days, respectively. Looking at the MBRA 337 scopoletin accumulation profile, it might be possible to talk about two peaks, one after one day and the second after six days.

Among the family K sample group, the REGWQ grouping test for scopoletin showed differences between PPD level groups (high, medium and low PPD response cultivars). The high PPD level group was separated from the rest from day one after harvesting. This separation was also observed when considering the area under the curve.

The other sample groups did not show such separation. REGWQ grouping for cultivars did not separate groups in samples processed at Bath, but separated cultivar MVEN 77 at days three and four in the December 1999 group. The same cultivar was also differentiated in the June 1999 group at days two, five and six. Peak frequencies for scopoletin in the Bath samples showed higher values for high PPD level at day two, medium level at day one and low level at day 6. The June 1999 group showed the peaks for high level at day two and medium and low at day five. The highest peak frequencies in December 1999 were at day one for high PPD level, and day two for low level.

Medium level cultivars presented the same peak frequency between days two and four (see appendices 8.2, 8.3, 8.4).

In general, the concentration graphs indicate higher values for scopoletin in high PPD levels along all sample groups. In addition, in all groups of samples and cultivars, it can be suggested that scopoletin and scopolin accumulate in higher concentrations compared to esculin and esculetin.

REGWQ grouping for scopolin only separated PPD level groups in the family K group. Low PPD level cultivars were differentiated from the other two groups in days three and four. REGWQ grouping at cultivar level only separated cultivar CM2177-2 at days three and five in the June 1999 group. The general behaviour of peak frequencies for scopolin showed higher values for high and medium PPD level groups between days three and four. Low PPD level groups presented the higher frequencies in the last days of the time course. The exception was family K group, where all PPD level groups peaked on the fourth day. Concentration graphs suggested the same patterns of frequency peak graphs, higher concentration values were present on the same day as the frequency of peaks.

REGWQ grouping for esculin separated PPD level groups in family K samples. From day two and including the area under the curve, low PPD level cultivars separated from high and medium levels. Cultivar separations were not observed. Peak frequency and concentration graphs showed the same patterns as scopolin, though esculin concentration was much lower than scopolin.

Finally, esculetin neither showed separation between PPD level groups or cultivars. Low PPD level cultivars, in Bath samples group, accumulated higher levels of esculetin throughout the storage time course compared with the other cultivars, and presented the higher frequency peak at five days after wounding stress.

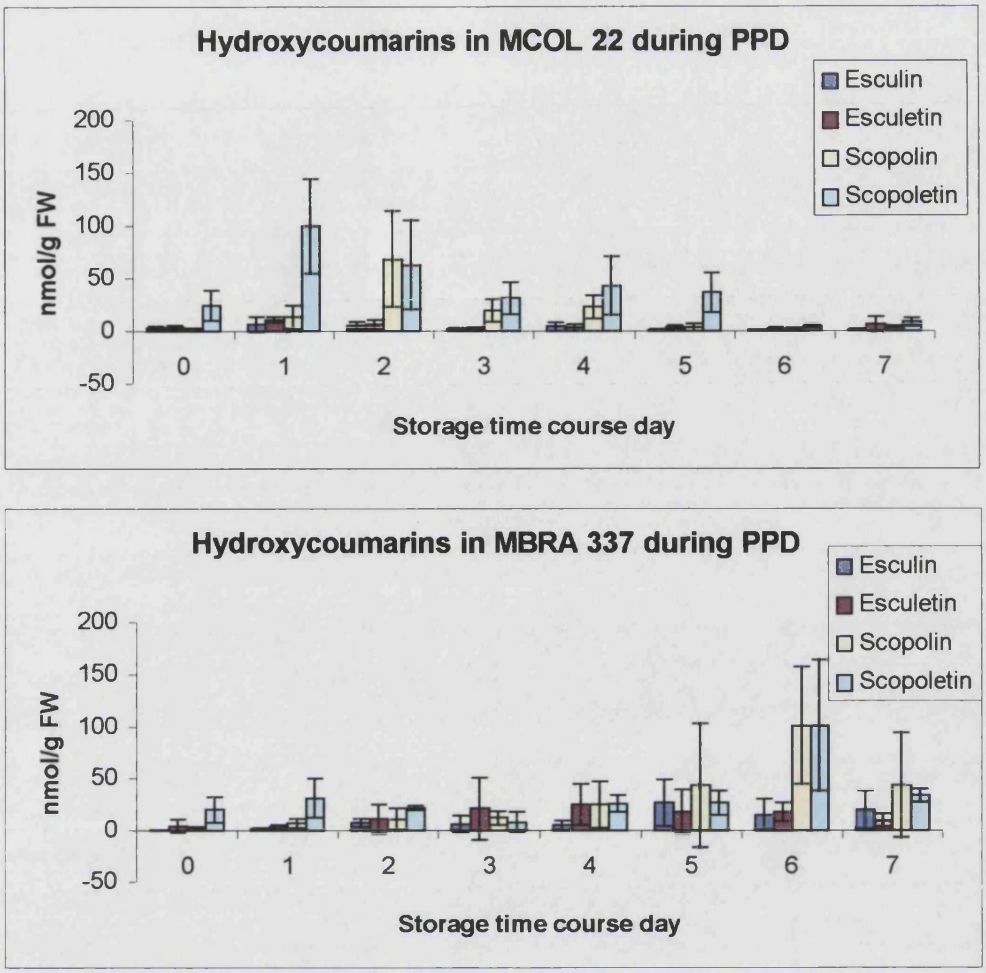


Figure 3.18. Hydroxycoumarins in cassava root extracts undergoing PPD in cultivars with contrasting responses towards PPD (low PPD, MBRA 337, and high PPD, MCOL 22). The results showed on this graph correspond to the group of samples processed at Bath.

3.2.6 Quantitative changes of flavan-3-ols

The quantitative changes in flavan-3-ols were measured only in the group of samples processed in Bath.

One example of the quantitative profile of (+)-catechin, (+)-gallocatechin and (+)-catechin gallate is shown in figure 3.19 (cultivar SM 985-9). Quantitative data for rutin and (-)-epicatechin were not analysed, because the HPLC peaks for those compounds were hardly detected in all extracts. As observed in all samples, there was a high variability in individual quantifications. Nevertheless, a tendency could be observed. During the first days the flavan-3-ols were detected at very low concentrations. After three days catechin and gallocatechin presented a slight increase. Two days later both metabolites showed a more significant increase, while catechin gallate still remained just detectable.

Looking at the complete set of data (Bath), the three flavan-3-ols were not synthesised *de novo*, but their concentrations immediately after wounding were very low. After four days of wounding the accumulation of the three metabolites was more pronounced, particularly for gallocatechin (appendix 8.4.1). As well, the peaks of the accumulating compounds were present after four days for the three susceptibility levels. In general, during the storage time course and especially after four days, the low susceptible cultivar MBRA 337 accumulated higher concentrations of flavan-3-ols compared to the other cultivars.

Despite the accumulation differences observed in the concentration and peaks graphs, the ANOVA analysis did not show significant differences between PPD levels or cultivars during the storage time course. Only two separations were observed between PPD level groups: low PPD level separated from the rest for catechin gallate at day seven and catechin at day four (appendix 8.3.1.1).

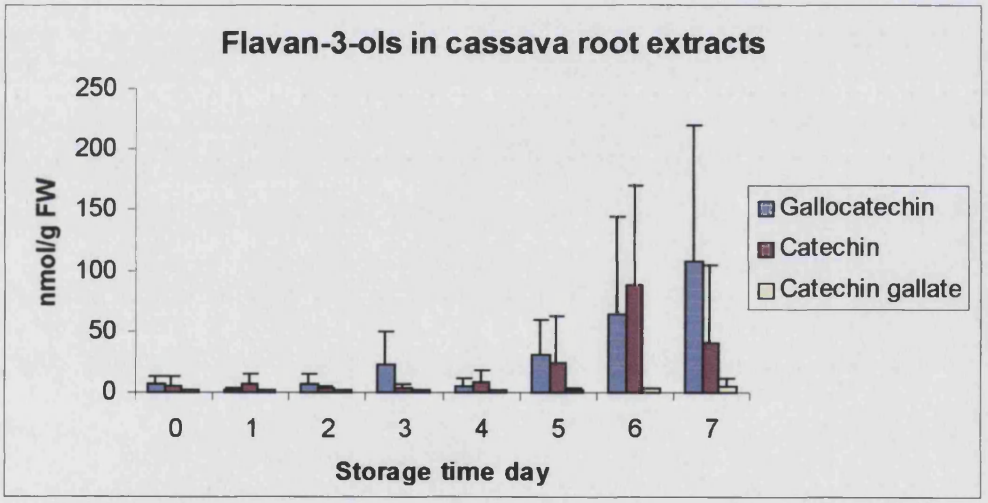


Figure 3.19. Flavan-3-ols in cassava root extracts undergoing PPD (cultivar SM985-9)

3.2.7 Quantitative changes of "PPD-marker"

Several attempts at the elucidation of the structure of the PPD related peak (PPD-marker, TLC R_f 0.66 and HPLC retention time 50 min) were not successful. The chemical nature of the compound still needs to be determined. Despite this, having an approximately idea of the accumulation of this compound might enable the usefulness of this compound as a biochemical marker for PPD to be determined. Bearing in mind that scopoletin has been determined as one of the secondary metabolites most related to PPD, we decided to quantify the "PPD marker" in terms of scopoletin. So the linear

regression equation used for quantifying scopoletin was also used for the PPD-marker. The PPD marker was quantified in all the groups of samples processed at CIAT.

Accumulation profiles of cultivars with contrasting PPD responses are presented in figure 3.20. As had been observed in all metabolite quantifications, "PPD-marker" quantification also showed a very high variability between replicates. Nevertheless, it was possible to see a trend in the accumulation of the PPD-marker peak. The compound was just detectable after one day of harvesting. The high susceptible cultivar, CM2177-2, showed larger PPD-marker amounts along the deterioration time course, principally when compared to the profile of the low PPD cultivar, MBRA 337. The general trend in PPD-marker accumulation was its increasing in concentration during the deterioration time course. Frequency peak graphs showed the highest values at day four (for all PPD levels) in family K and December 1999 groups. June 1999 samples showed the highest frequency peaks at day six for low and high PPD levels, and at day 4 for the medium level. Concentration graphs showed the largest accumulation of the PPD-peak at the same days as those at which the high frequency peaks occurred. REGWQ grouping by levels did not find differences in samples of the June 1999 group. Among the December 1999 samples, high PPD cultivars were differentiated from the rest at day three. As well, considering the area under the curve, high PPD and low PPD cultivars were considered as different groups. REGWQ grouping by cultivar significantly separated MDOM 5 from the low cultivars MBRA 337 and MBRA 12. At day two REGWQ grouping clearly separated SM 985-9, one of the most susceptible cultivars, from the three low PPD cultivars. In the family K group of samples, low PDD level cultivars were differentiated from the rest from day two.

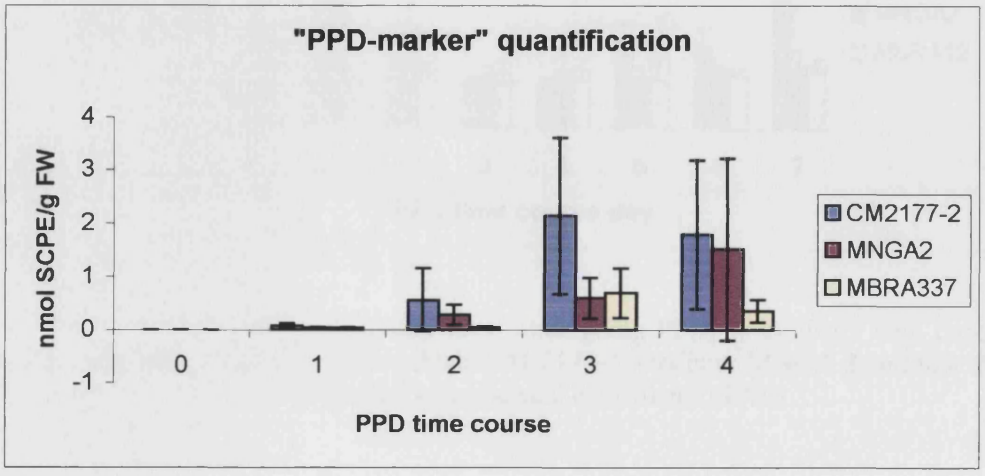


Figure 3.20. "PPD-marker" quantification in cassava root ethanolic extracts of cultivars with contrasting responses to PPD (high: CM 2177-2, medium: MNGA 2 and low: MBRA 337)

3.2.8 Quantitative changes of tannins

Quantitative changes of tannins were measured in the group of samples processed at CIAT in June 1999.

Tannin accumulation expressed in tannic acid equivalents (TAE) of three cultivars, CM 2177-2, MNGA 2 and MBRA 337, with contrasting responses to PPD, high, medium and low respectively, is shown in figure 3.21. Tannins concentration during the post harvest storage time course did not reveal a marked trend. Cultivar CM 21777-2 showed a slight increase in concentration from day five, while the concentrations of MBRA 12 were more or less the same throughout the time course. MNGA 2 showed a peak on the first day, but the variation between repetitions was very high. The graphs for tannin concentration between PPD levels revealed that the low PPD cultivars accumulated slightly higher quantities throughout the storage course. However, the ANOVA analysis did not separate the different PPD level groups. Looking at the differences between cultivars, the ANOVA separated MCOL 22 from the other at days 0, 5 and 7. Additionally, MCOL 22 presented the highest mean values during the time course (appendix 8.2, 8.3). With respect to graph peaks, only one significant peak was observed for high PPD level at day seven (appendix 8.4.2)

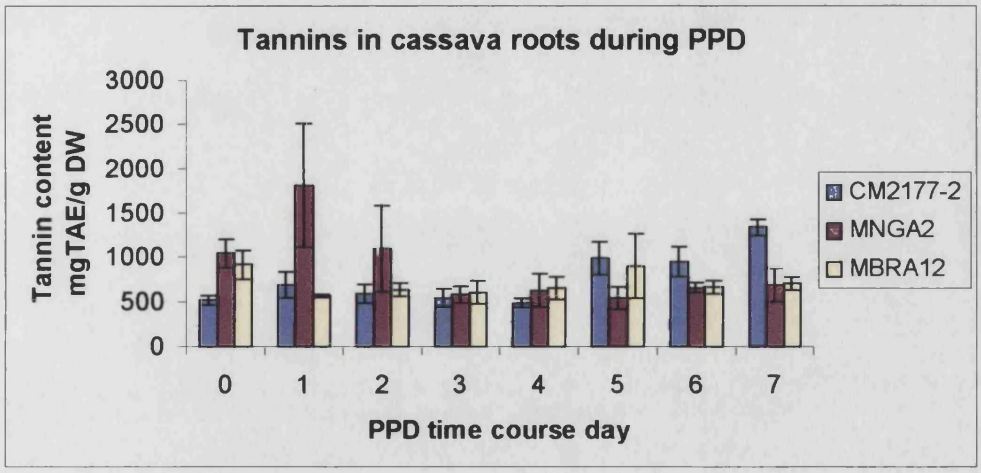


Figure 3.21. Tannin content in cassava roots undergoing PPD, in cultivars with contrasting responses to post-harvest deterioration (high: CM 2177-2, medium: MNGA 2 and low: MBRA 12). Tannin concentration is expressed in tannic acid equivalents (TAE)

3.2.9 Principal component analysis

The principal component analysis (PCA) was conducted in order to study the separation of cultivars during the PPD time course considering all secondary metabolites in the analysis. We wanted to determine the key metabolites of the PPD response, i.e. the metabolites determining the separation of the cultivars in PPD-levels. However, this could not be achieved because clusters identified through the analysis were composed of a mixture of cultivars from different PPD levels. The number of clusters were determined with 90 % similarity.

The results of the PCA analysis are shown in appendix 8.6.1 and in figure 3.22. Figure 3.22 represents three-dimensional graphs showing the relationships of cassava cultivars after secondary metabolites measurements.

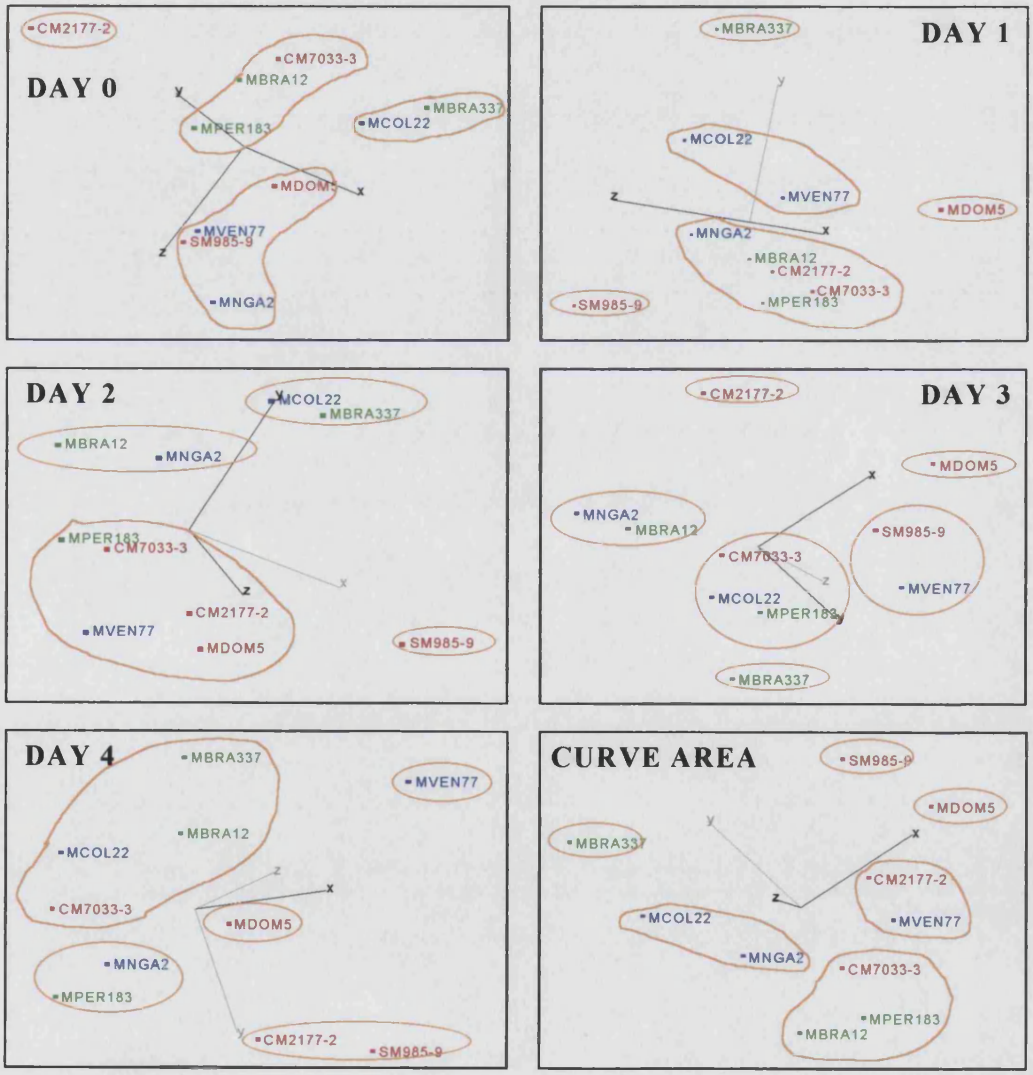


Figure 3.22. Three-dimensional graph from a Principal Component Analysis showing relationships of cassava cultivars after secondary metabolites measurements day by day and area under the curve.

3.2.10 Localisation of secondary metabolites in roots

3.2.10.1 Localisation of lignin and suberin

Staining of cassava roots slices with phloroglucinol and sudan III resulted in the localisation of lignin and suberin, respectively, in the vascular parenchyma. Figure 3.23a shows microscopic images of cross sections of cassava root stained for lignin. The accumulation of lignin around the vascular bundles was very clear. Differences in lignin accumulation during the post harvest time course or between cultivars were not observed (Fig. 3.23b).

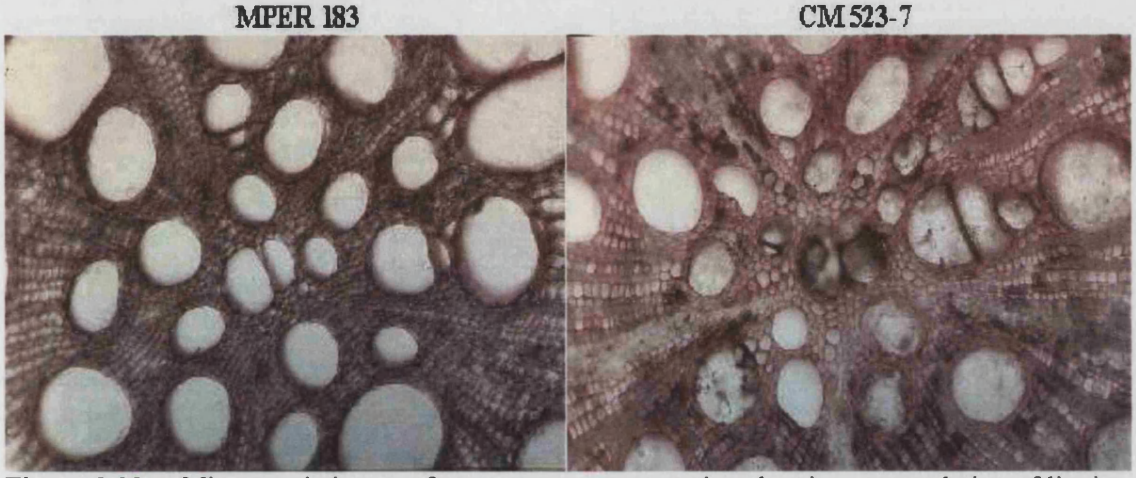


Figure 3.23a. Microscopic image of cross cassava root section showing accumulation of lignin around vascular bundles. Images correspond to cultivars MPER 183 (low PPD) and CM 523-7 (high PPD). (x100)

3.2.10.2 Localisation of callose and flavonoids.

Localisation of callose and flavonoids in cassava root slices was not possible. The reason was the interference of the blue fluorescence characteristic of coumarins present in roots with the yellow-orange fluorescence shown by the stained callose and flavonoids. Figures 3.24 and 3.25 show root slices stained for callose and flavonoids visualised under long UV light.

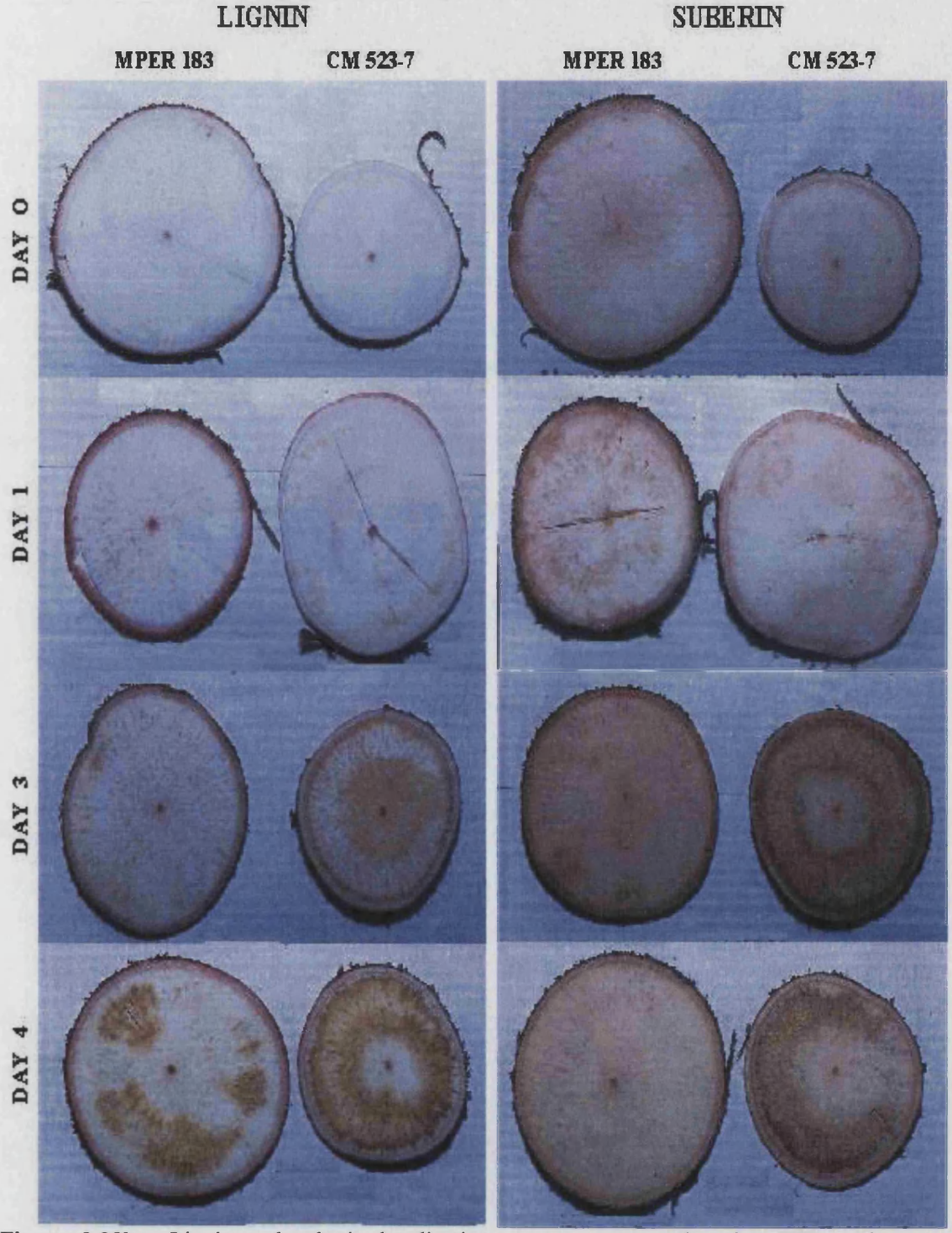


Figure 3.23b. Lignin and suberin localisation cassava roots undergoing PPD. Pictures correspond to cultivars MPER 183 (low PPD) and CM 523-7 (high PPD), during a time course of four days.

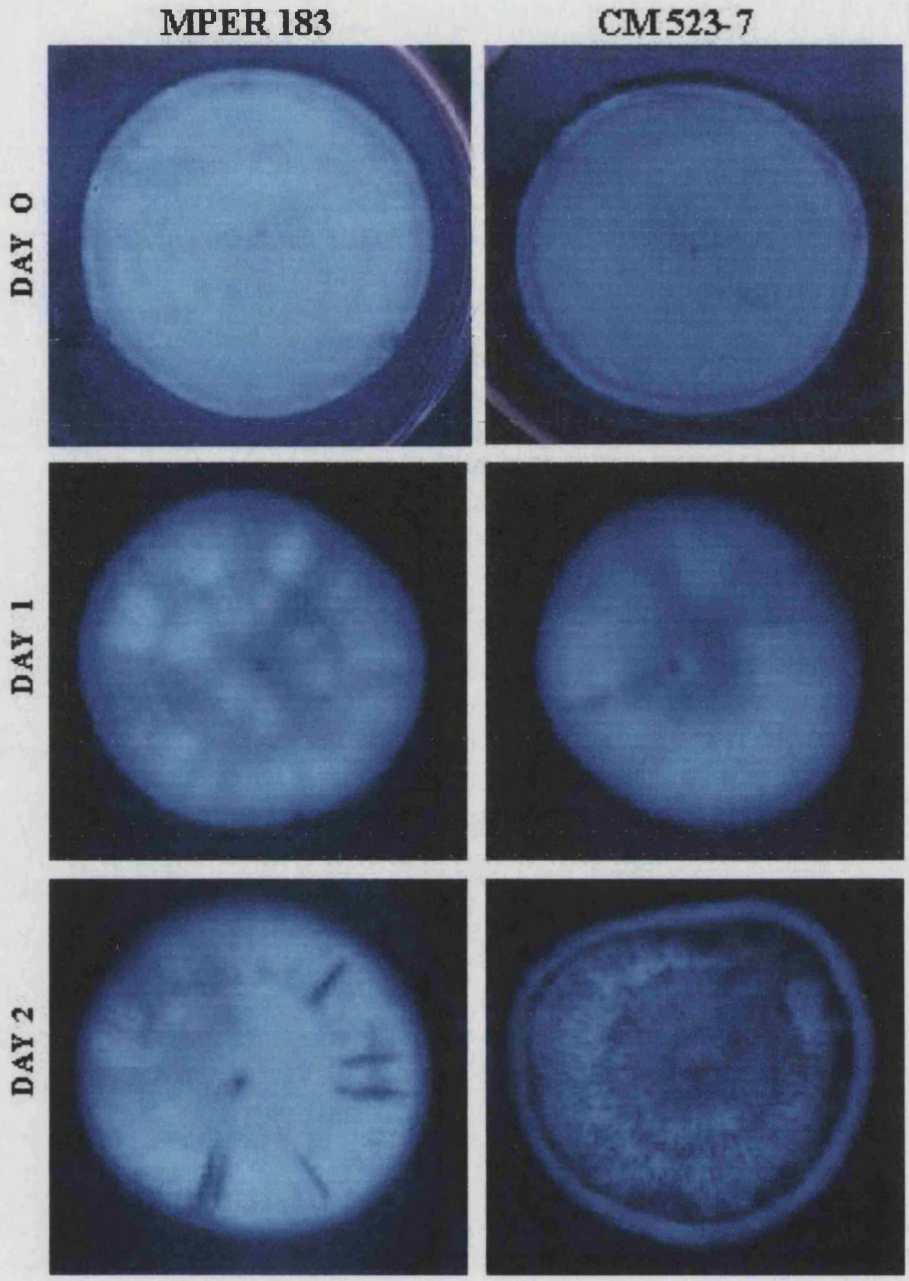


Figure 3.24. Cassava root slices stained for callose localisation. Pictures correspond to cultivars MPER 183 (low PPD) and CM 523-7 (high PPD) visualised under UV_{366nm}

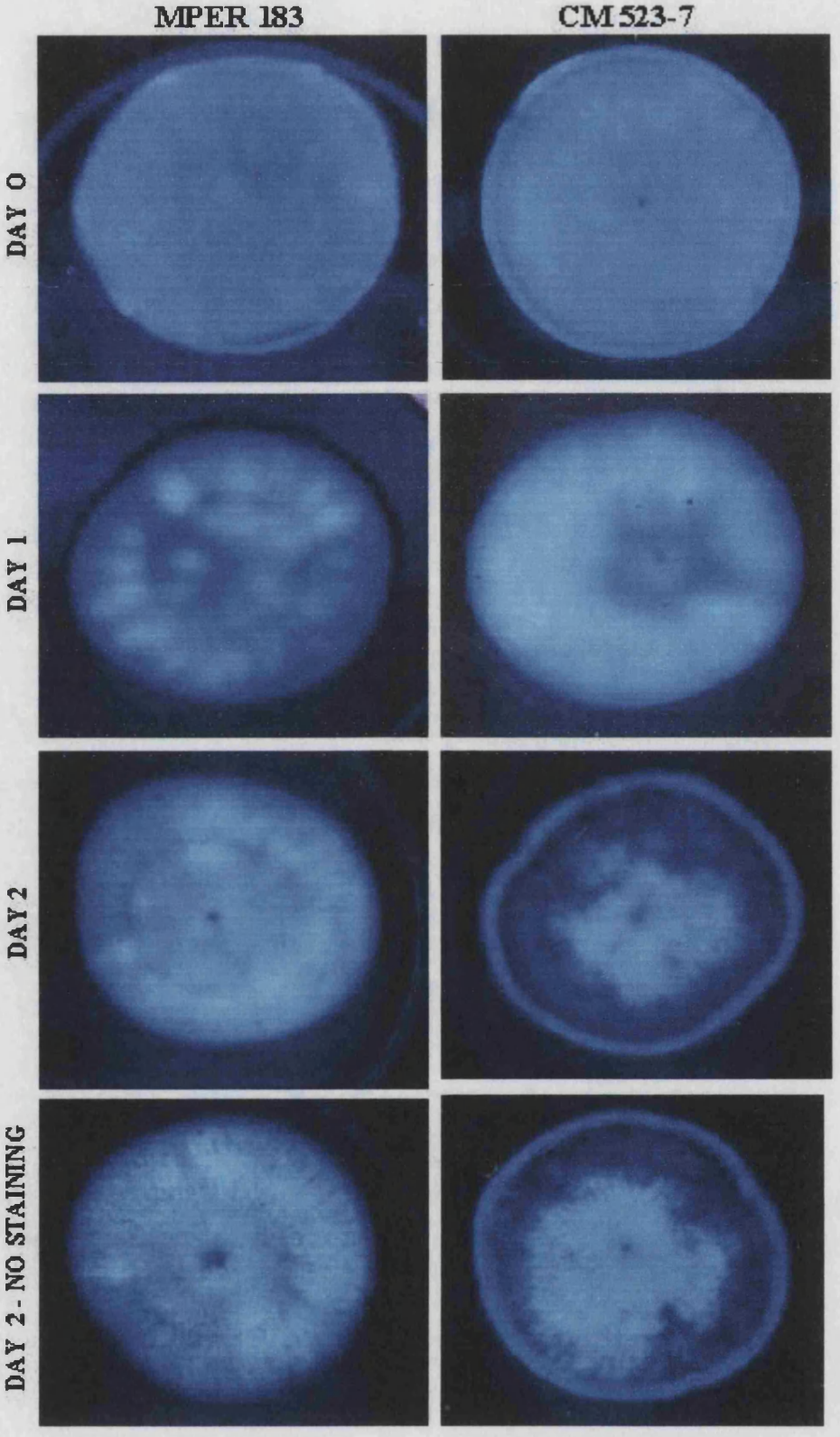


Figure 3.25. Cassava root slices stained for flavonoids localisation. Pictures correspond to cultivars MPER 183 (low PPD) and CM 523-7 (high PPD) visualised under UV_{366nm}

3.2.11 Biological activity of secondary metabolites present in cassava roots during PPD.

Different bioassays were performed with the aim of determine the anti-microbial activity of the secondary metabolites produced in response to PPD. The data presented correspond to ethanolic root extracts from commercial roots obtained from a supermarket in Bristol.

3.2.11.1 TLC bioautography

As mentioned in chapter 2.4.3 the non polar fraction of the ethanolic root extracts (extracted by SPE) was separated in TLC plates and then sprayed with conidial suspensions. After incubation for four days, growth inhibition of *T. harzianum* (Fig. 3.26) was detected. This zone of inhibition indicated the presence of an anti-fungal compound. Observing the inhibition area during the storage time course, a positive relationship between concentration of the antifungal compound and storage time course could be observed.

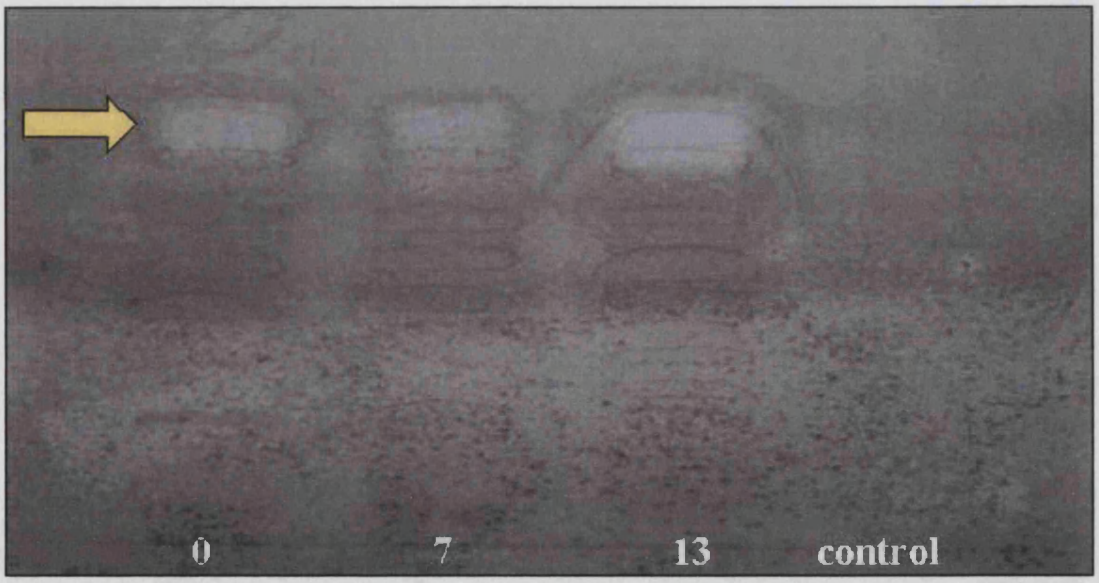


Figure 3.26: TLC bioautography for anti-microbial compounds from cassava ("Bristol") ethanolic root extracts (non polar phase from solid phase extraction) during storage time course. Biological activity determined by means of *T. harzianum* conidia suspension. Volume of extract loaded equivalent to 2 g fresh weight of root tissue. The yellow arrow indicates the biological active band.

In a second assay, ethyl acetate and butanol fractions of the ethanolic cassava root extracts separated by LPE were also run in TLC plates. This time, aliquots corresponding to different concentrations of a bulk of crude ethanolic extracts were used to try to determine the extract minimal inhibitory concentration. Fungal growth

inhibition (Figure 3.27) in the ethyl acetate phase suggested these anti-microbial compounds had medium polarity. The bioautographies with *F. avenaceum* and *C. cucumerimnum* showed four inhibiting bands (Figure 3.27). One of these bands was identified as scopoletin by comparing its retention time with that of a reference loaded onto the TLC plate (R_f 0.65). Relative retention times index based on scopoletin were determined for the other three bands of 1.05, 1.14 and 1.18. No inhibition of growth of *Staphylococcus aureus* with the non polar and medium polar phases was detected.

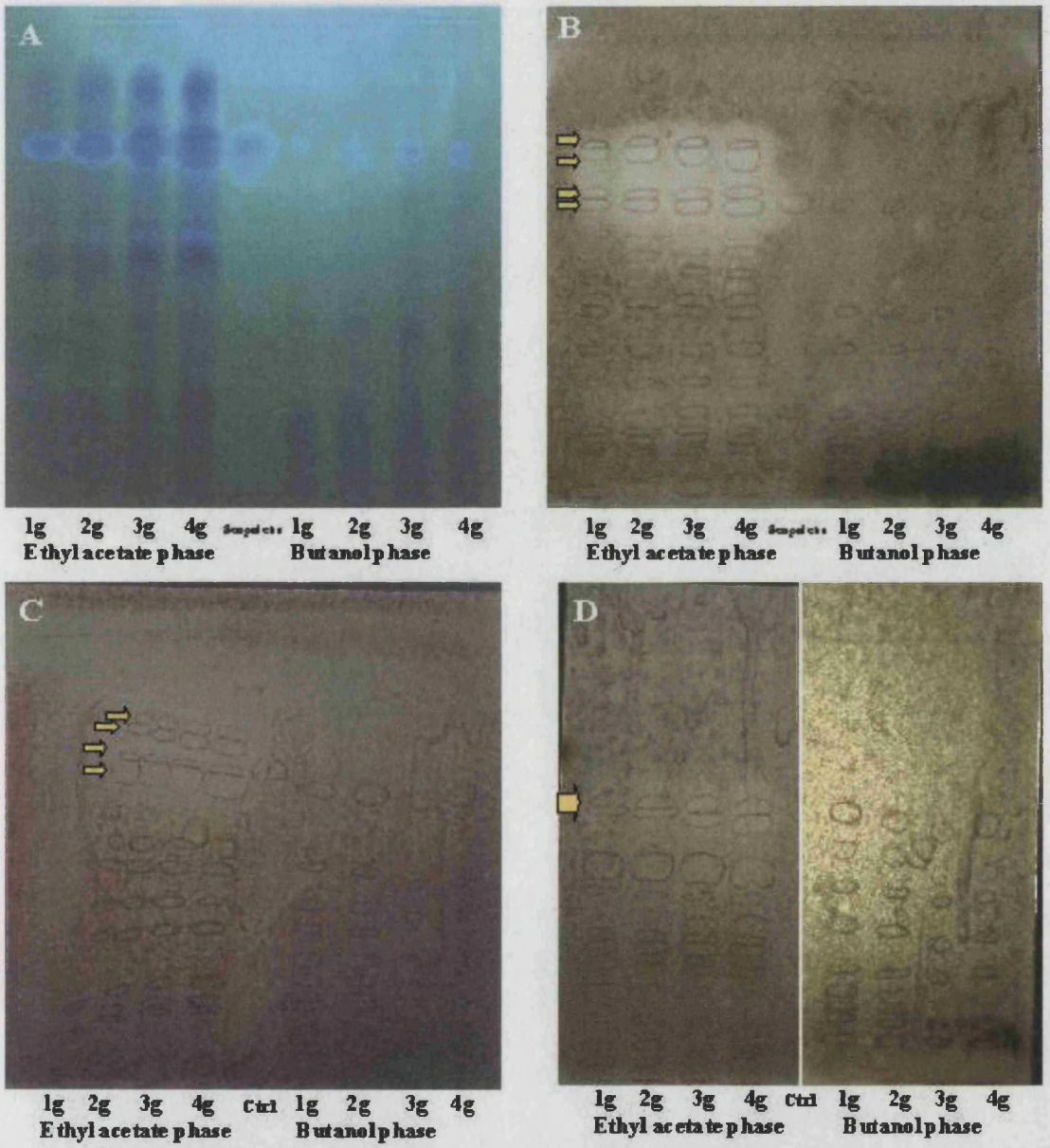


Figure 3.27. A) TLC pattern of secondary metabolites (detection at 254nm) from ethanolic extracts (non-polar fractions) in a range of concentration (based on fresh weight). TLC bioautography showing the anti-fungal activities of secondary metabolites present in cassava roots undergoing PPD. B) C. *cucumerinum*, C) *F. avenaceum*, and D) *T. harzianum*. The yellow arrows indicate the anti-microbial bands.

Inhibition of fungal and bacterial growth observed in figures 3.28 and 3.29 suggested the presence of biological active compounds with high polarity. It was not possible to determine the number of anti-microbial compounds present in the polar fraction of the ethanolic extracts because no solvent system tested showed appropriate resolution of these bands.

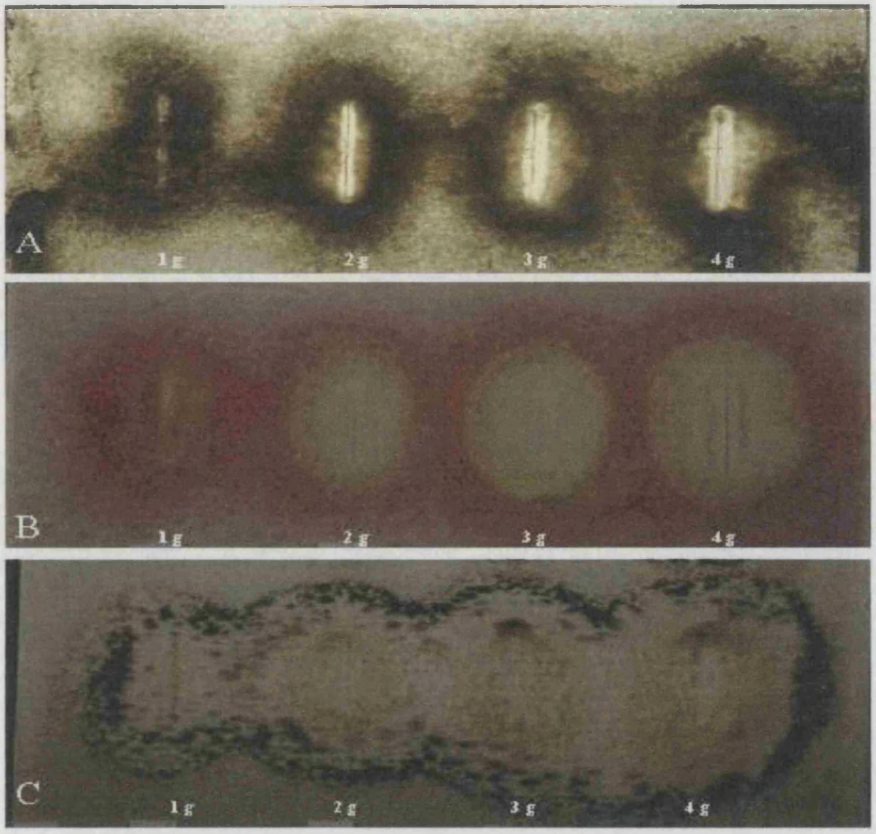


Figure 3.28: TLC Bioautography showing the anti-fungal activity of the ethanolic root extract (polar fraction) in a concentration range (based in fresh weight). The micro-organisms used were: A) *C. cucumerimun*, B) *F. avenaceum* and C) *T. harzianum*.

Figure 3.29. TLC Bioautography showing the anti-bacterial activity of the ethanolic root extract (polar fraction) in a concentration range (based in fresh weight). The plate was laid over with nutrient agar containing a suspension of *S. aureus*.

3.2.11.2 Agar Well Diffusion Assay

Growth inhibition of *T. harzianum* around the wells was not observed. Probably it was due to low sensitivity of the micro-organism or low diffusion of the crude extract into the agar.

3.2.11.3 Fungal Minimal Active Concentration Determination

The minimal concentration of extract for growth inhibition detection, tested with all micro-organisms used for the bioassays, was not determined since inhibition with the most dilute sample (volume equivalent to 1 g FW) was detected.

The determination of the minimal active concentration for the reference compounds present in cassava and the other compounds known to be anti-microbials was not clear (Fig. 3.30). Growth inhibition of the three fungi was only observed with the highest concentration used (100µmol) of scopoletin and naringenin. This could indicate very low sensitivity of the fungi used for the bioassays. Notwithstanding, it was expected to see fungal growth inhibition with lower concentrations based on the measurements obtained for concentrations of scopoletin in extracts of cassava roots undergoing PPD.

3.2.12 Relationship between carotene content, dry matter and PPD

Relationships between carotene content, PPD and dry matter were calculated among the progeny of four crosses: Family CM9726 (MDOM 5, female, low PPD x CM523-7, male, high PPD) with 29 progeny; Family CM9679 (SM1551-18, female, white x CM8371-7, male, yellow) with 33 progeny; Family CM9680 (HMC1, female, white x CM8371-7, male, yellow) with 49 progeny; Family CM9681 (CM8371-7, female, yellow x MPER 183, male, white, low PPD) with 21 progeny. The carotene content was determined following the protocol proposed by (Safo-Katanga et al. 1984). The crude data for carotene content, dry matter and PPD response were provided by the cassava breeding team at CIAT.

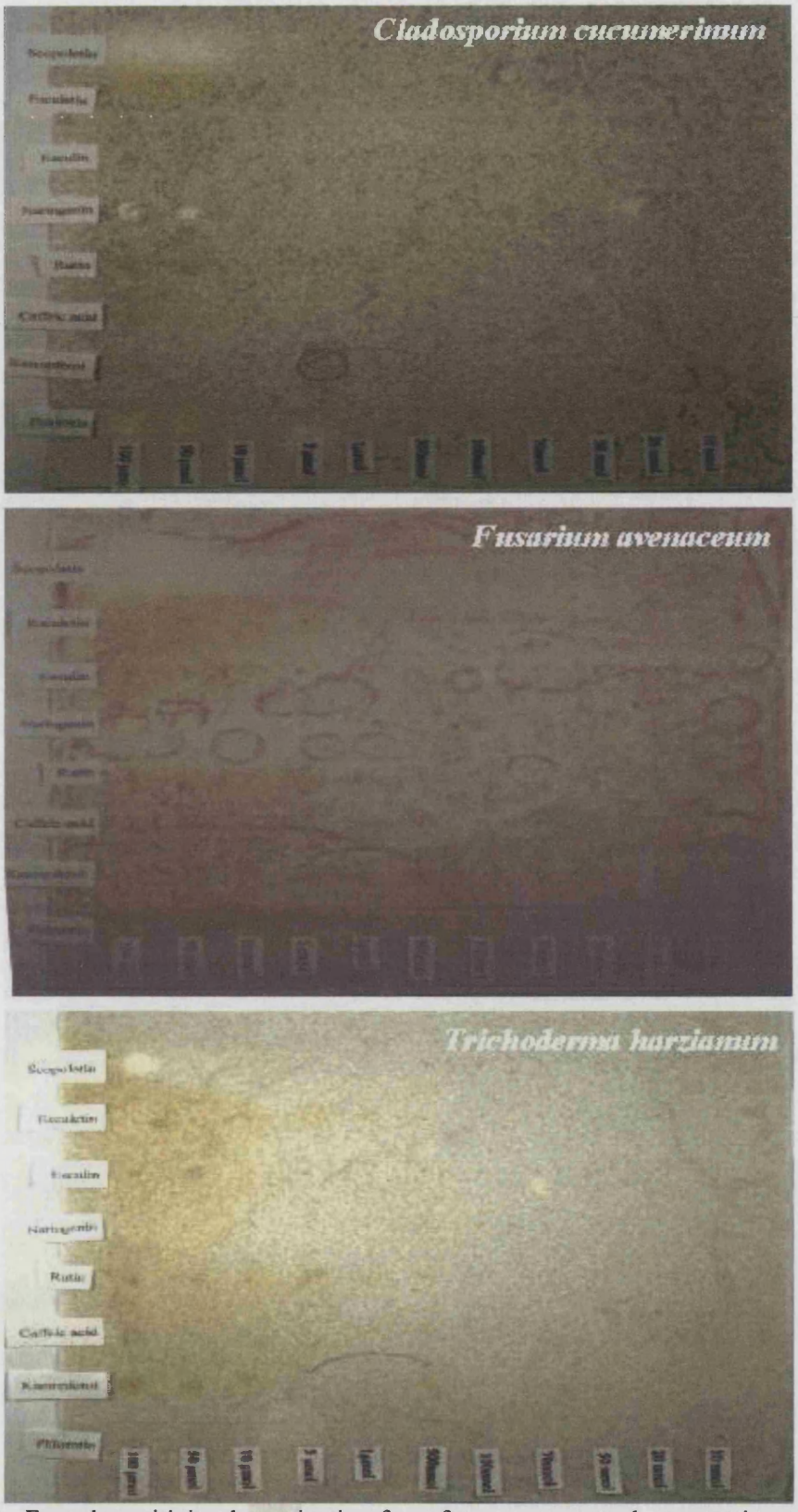


Figure 3.30. Fungal sensitivity determination for reference compounds present in cassava and others known as phytoalexins in a wide range of concentrations.

Table 3.3 shows the results of the correlations between carotene content, dry matter and PPD for the four families and for all genotypes combined from the four families. Correlation coefficients in bold represent significant probabilities (<0.05) and underlined correlation coefficients represent highly significant probabilities (<0.001). Results showed that there was a significant correlation between PPD and dry matter content for all families. In the CM9680, there was a negative and significant correlation between carotene content and PPD.

CM 9679			
	PPD	DM	CAROT
PPD	1	**0.67541**	-0.13321
	0	0.0001	0.4599
DM	**0.67541**	1	-0.03755
	0.0001	0	0.8357
CAROT	-0.13321	-0.03755	1
	0.4599	0.8357	0

CM 9680			
	PPD	DM	CAROT
PPD	1	**0.61626**	**-0.33744**
	0	0.0001	0.0218
DM	**0.61626**	1	**-0.32682**
	0.0001	0	0.0266
CAROT	**-0.33744**	**-0.32682**	1
	0.0218	0.0266	0

CM 9681			
	PPD	DM	CAROT
PPD	1	**0.53064**	-0.11921
	0	0.0133	0.6068
DM	**0.53064**	1	-0.36837
	0.0133	0	0.1004
CAROT	-0.11921	-0.36837	1
	0.6068	0.1004	0

CM 9726			
	PPD	DM	CAROT
PPD	1	**0.35921**	0.13416
	0	0.0195	0.397
DM	**0.35921**	1	-0.12025
	0.0195	0	0.4481
CAROT	0.13416	-0.12025	1
	0.397	0.4481	0

ALL GENOTYPES			
	PPD	DM	CAROT
PPD	1	**0.52685**	0.01433
	0	0.0001	0.8656
DM	**0.52685**	1	**-0.2201**
	0.0001	0	0.0085
CAROT	0.01433	**-0.2201**	1
	0.8656	0.0085	0

Table 3.3. Correlation analysis between PPD, carotene content and dry matter for four families and for all genotypes from the four families. The correlation coefficient and the associated probability are indicated.

In another study, 500 genotypes from the elite collection were assayed for carotene content and PPD response. A negative correlation was observed between the two traits (correlation coefficient: -0.1125, probability: 0.0001). The correlation coefficient was not very high, but it may be suggested that high carotene contents may prevent the PPD-response (fig. 3.31). All cultivars with a high carotene content showed low PPD

responses. The correlation between PPD and dry matter was also calculated in this group of genotypes (fig 3.32). The results reconfirmed the positive correlation between PPD and dry matter content (correlation coefficient: 0.4302, probability: 0.0001).

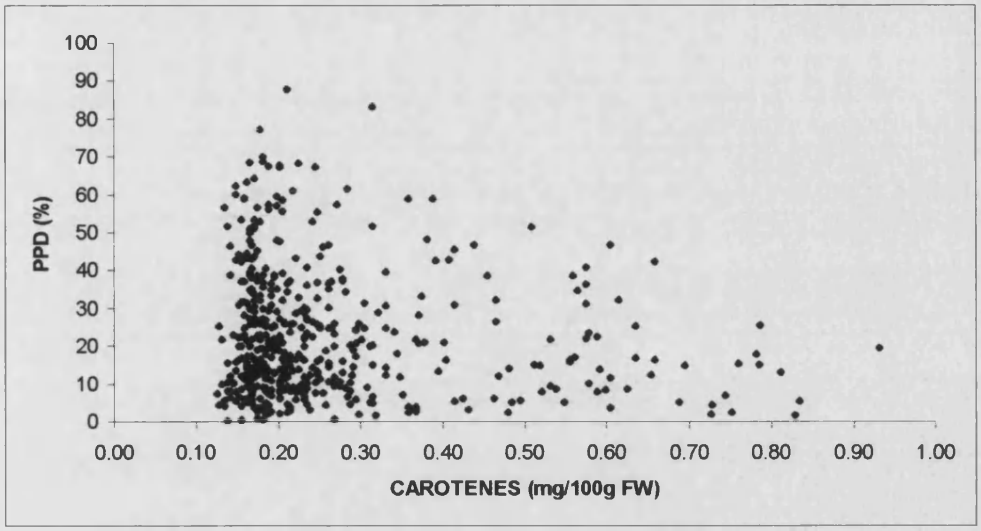


Figure 3.31 Correlation between carotene content and PPD in 500 cassava genotypes of the elite collection held at CIAT.

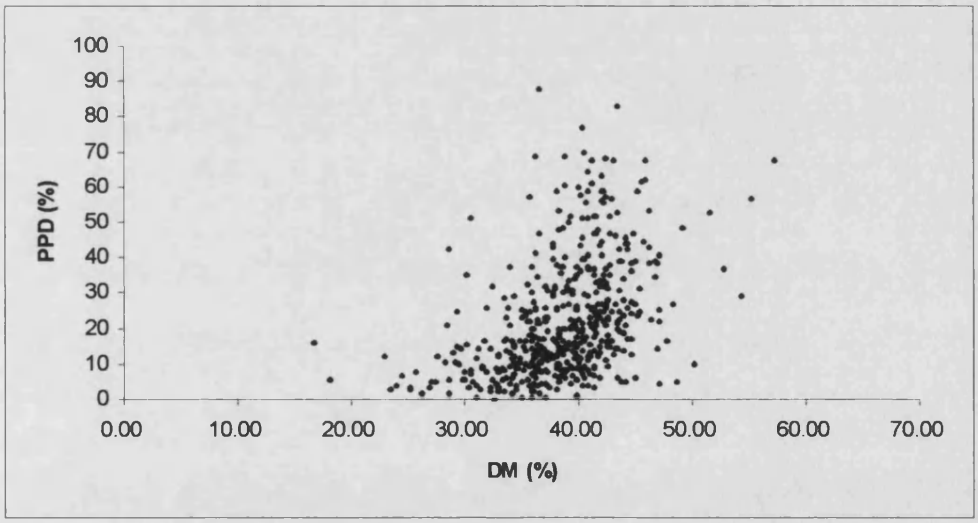


Figure 3.32 Correlation between dry matter content and PPD in 500 cassava genotypes of the elite collection held at CIAT.

3.3 DISCUSSION

The results presented here describe the identification, quantification and localisation of secondary metabolites. Based on the results presented in this study, the secondary metabolites, most correlated with PPD response were the hydroxycoumarins. Among

the hydroxycoumarins, a new secondary metabolite, esculetin, associated and highly correlated with the PPD response was identified in this study. However, the accumulation of this metabolite was low in almost all cultivars of the Bath group of samples and could not be detected in other sample groups. It can be suggested that this metabolite could be detected in the Bath group of samples because these cultivars were "over-stressed" after the storage time course. Changing the gradient of the HPLC solvent system might help detecting esculetin in all samples and cultivars.

Tables 3.4 and 3.5 summarise the results obtained for the average of metabolites concentration and for the frequency of peaks for the three PPD susceptibility levels respectively. These tables can help to identify the best day to evaluate the most important secondary metabolites associated with the PPD response, having in mind that one of the project aims is the development of screening methods for use in germplasm evaluation of breeding programmes. For scopoletin and scopolin, days three and four, respectively, reflect the general behaviour of cultivars, especially those in the high PPD level. The other hydroxycoumarins did not accumulate in detectable levels in most of the groups of samples and the rest of secondary metabolites, except the "PPD marker", were essentially associated with a microbial deterioration. For the PPD marker, the best day to evaluate is day four as tables 3.3 and 3.4 show.

This study also presented an attempt to perform a time course experiment of the localisation of lignin and suberin. However, the results showed localisation around the xylem vessels, there were no differences over time in the localisation of these secondary metabolites.

Biological activity of secondary metabolites present in cassava root ethanolic extracts, as previously determined by (Taniguchi et al. 1984) was confirmed. Scopoletin proved as an antimicrobial compound for several fungal species but when bacteria where assayed, no antimicrobial activity was detected. Three TLC bands proved to have antimicrobial activity but efforts to determine their chemical nature have to be made in order to a further characterization of these unknown metabolites. Other secondary metabolites present in cassava root ethanolic extracts detected by TLC showed an antioxidant activity. Scopoletin was one of them, the rest remain to be determined.

METABOLITES CONCENTRATION DURING DETERIORATION AMONG PPD LEVELS												
SAMPLE GROUP	TIME COURSE DAY	SCOPOLETIN	SCOPLIN	ESCULETIN	ESCULIN	CATECHIN	GALLOCATECHIN	CATECHIN GALLATE	PPD-M	PHENOLIC CONTENT	TANNIN CONTENT
BATH	0								NE	NE	NE
	1								NE	NE	NE
	2	M-H							NE	NE	NE
	3		M						NE	NE	NE
	4			L					NE	NE	NE
	5		H	M-H	L-M		M		NE	NE	NE
	6	L	L			M-H	L-H	H	NE	NE	NE
	7				H	L		L-M	NE	NE	NE
JUNE 1999	0			0	0	NE	NE	NE		L-M-H	L
	1			0	0	NE	NE	NE			M
	2	L		0	0	NE	NE	NE			
	3		H	0	0	NE	NE	NE			
	4			0	0	NE	NE	NE	M		
	5		M	0	0	NE	NE	NE	H		
	6	M	L	0	0	NE	NE	NE			
	7	H		0	0	NE	NE	NE	L		H
DEC 1999	0			0		NE	NE	NE			NE
	1			0		NE	NE	NE			NE
	2			0		NE	NE	NE		M	NE
	3		M-H	0	H	NE	NE	NE	H		NE
	4	L-M-H	L	0	L-M	NE	NE	NE	L-M	L-H	NE
FAMILY K	0			0		NE	NE	NE			NE
	1			0		NE	NE	NE			NE
	2	H		0		NE	NE	NE			NE
	3	L-M		0		NE	NE	NE			NE
	4		L-M-H	0	L-M-H	NE	NE	NE	L-M-H	L-M-H	NE

Table 3.4 Summary of the results obtained for the average of concentration metabolites for the three PPD susceptibility levels: high (**H**), medium (**M**) and low (**L**). The position of the convention used to designate the PPD level indicates the day of the storage time course at which the maximum concentration of secondary metabolite is reached.
NE:metabolite not evaluated.
0: metabolite not detected.

FREQUENCY OF PEAKS DURING DETERIORATION AMONG PPD LEVELS											
SAMPLE GROUP	TIME COURSE DAY	SCOPOLETIN	SCOPLIN	ESCULETIN	ESCULIN	CATECHIN	GALLOCATECHIN	CATECHIN GALLATE	PPD-M	PHENOLIC CONTENT	TANNIN CONTENT
BATH	0	M						M	NE	NE	NE
	1	M		H		M			NE	NE	NE
	2	H	M			M			NE	NE	NE
	3		M	L		M			NE	NE	NE
	4		H	L		L-M		L	NE	NE	NE
	5				L-M	M	M		NE	NE	NE
	6	L	L	L-M		L-M-H	H	L	NE	NE	NE
	7		M	H	M-H	L	L-M	L-M-H	NE	NE	NE
JUNE 1999	0			0	0	NE	NE	NE		L-M-H	
	1			0	0	NE	NE	NE		L-H	M
	2	L		0	0	NE	NE	NE			
	3			0	0	NE	NE	NE			
	4		L	0	0	NE	NE	NE	M	H	L
	5	M-H	M	0	0	NE	NE	NE		H	
	6		M-H	0	0	NE	NE	NE	L-H	M-H	L
	7		M	0	0	NE	NE	NE		H	H
DEC 1999	0			0		NE	NE	NE			NE
	1	H		0		NE	NE	NE			NE
	2	L-M		0		NE	NE	NE		M	NE
	3	M	M-H	0	M-H	NE	NE	NE	H	H	NE
	4	M	L	0	L	NE	NE	NE	L-M-H	L-M	NE
FAMILY K	0			0		NE	NE	NE			NE
	1			0		NE	NE	NE			NE
	2	H		0		NE	NE	NE			NE
	3	L-H		0	M-H	NE	NE	NE			NE
	4	L-M	L-M-H	0	L-H	NE	NE	NE	L-M-H	L-M-H	NE

Table 3.5 Summary of the results obtained for the frequency of peaks for the three PPD susceptibility levels: high (**H**), medium (**M**) and low (**L**). The position of the convention used to designate the PPD level indicates the day of the storage time course at which the highest frequency of peaks is reached. There are cases in that the highest value of peaks frequency is the same for different days of the storage time course.
NE:metabolite not evaluated.
0: metabolite not detected.

PPD showed a negative correlation with carotene content and a positive correlation with dry matter. The close association between dry matter (a desirable characteristic) and the PPD response, together with the environmentally induced variability of the PPD response, suggest the difficulty of breeding for delayed PPD. Even though the value of the correlation coefficient between carotene content and PPD is low, the PPD response for those cultivars with over 0.5 mg carotene/100 g fresh weight is low. This may suggests, that there is a threshold value beyond which this antioxidant plays a role in modulating the PPD response. The negative effect of antioxidants on PPD occurrence was also confirmed by (Campos and de Carvalho 1992). High levels of ascorbic acid (40 mg/100 g FW) in roots reduced oxidations and consequently the PPD damage.

CHAPTER 4

ENZYMATIC ACTIVITY AND OTHER WOUND RELATED RESPONSES INVOLVED IN PPD

4 ENZYMATIC ACTIVITY AND OTHER WOUND RESPONSES INVOLVED IN PPD

4.1 INTRODUCTION

Mechanical wounding in plants provokes a chain of defence responses, which are characterised by the activation of defensive related genes and the expression of diverse proteins that play determinant roles in pathogen attack and wound healing.

Reactive oxygen species (ROS) are generated in plants in response to wounding. They are key components in diverse physiological process: a) transmembrane signalling and induction of formation transfer, respiratory burst, local defence systems and systemic resistance; b) cell, tissue and organ damage due to reductive oxygen activation in almost all cellular compartments; c) defence reaction in the apoplast; d) light dependent damage and senescence; e) release of nitric oxyde and interaction with superoxide producing peroxynitrile; and f) formation of hormone-like messengers from jasmonic acid (reviewed by Hippeli et al. 1999). ROS have been also involved in lignification and suberisation (Bernards et al. 1999).

The main forms of ROS are singlet oxygen ($^1O_2$), the superoxide radical ($O_2 \cdot^-$), the hydroperoxyl radical ($HO_2\cdot$), and hydrogen peroxide H_2O_2. H_2O_2, the most stable of the reactive oxygen intermediates, has been associated in the cross-linking of the cell wall proteins (Bradley et al. 1992). The generation of H_2O_2 in response to wounding has been proved in various species (Orozco-Cardenas and Ryan 1999). In some species (such as cucumber) the response was located primarily at wound sites, whereas in others (like maize, cotton and potato) the response was strongly systemic.

Catalase modulates the breakdown of hydrogen peroxide to water and molecular oxygen and is widespread all over the aerobic organisms. A possible role for catalases during PPD was first studied by Czyhrinciw and Jaffé (1951), who proposed catalases, peroxidases and dehygrogenases as enzymes related with this process. Aside from the study by Czyhrinciw and Jaffé and the characterisation of a cassava catalase (MecCAT1) by Reilly et al. (2001), no further studies on catalases during PPD have been reported.

Catalases of higher plants play multiple roles in resistance to oxidative stress, photorespiration and germination. In addition, catalase has been proposed to mediate in signal transduction involving H_2O_2 as a second messenger (Ryals et al. 1994). Catalase

has been involved in hypersensitive responses and systemic acquired resistance (Ryals et al. 1994).

Oxidative processes involving phenolic compounds may modify the quality of fresh fruits and vegetables. Enzymatic browning is one the key oxidative events inducing the development of unattractive colour, flavour and loss of nutrients. In the large group of oxidoreductases, polyphenol oxidases and peroxidases, are involved in the oxidative degradation of phenolics. Polyphenol oxidases have been related mainly to discoloration and browning of fruits and vegetables (Amiot et al. 1997).

Polyphenol oxidases are copper proteins that include three types of enzymes depending on the substrate: a) monophenol oxidase (EC 1.14.18.1) or cresolase, b) o-diphenol oxidase (EC 1.10.3.1) or catecholase or phenolose and c) p-diphenoloxidase (EC 1.10.3.2) or laccase (Amiot et al. 1997). The activities of polyphenol oxidases are the hydroxylation of monophenols to o-diphenols, and the oxidation of the o-diphenols to quinones, which are powerful oxidising agents (Barz and Koster 1981). The quinones or polymer products derived from PPO activity form the brown pigments observed in many crops after injury or harvest. Polyphenol oxidase is also activated in response to wounding (Constabel et al. 1995)

Peroxidases have been extensively studied because they catalyse many important reactions and have a complex isozyme structure. These reactions comprise: a) oxidation of substrates with H_2O_2 (peroxidase reactions), b) introduction of oxygen into a substrate (oxygenase reaction), c) electron transfer reactions (oxidase reaction), d) transalkylation and e) halogenation reactions (Barz and Koster 1981).

In general the peroxidase reaction may be summarised as: $H_2O_2 + 2HA \rightarrow 2H_2O + 2A$, where HA represents the electron donor. The substrate specificity of peroxidases is relatively low, whereas their specificity for H_2O_2 is high (Strack 1997).

Peroxidases can be divided in two groups. The first group comprises peroxidases which primary function is the scavenging of H_2O_2 or organic hydroperoxides. They are known as ascorbate, gluthathione, cytochrome C or NADH peroxidases. The second group is constituted by the guaiacol peroxidases (EC 1.11.1.7), oxidoreductases. They are named guaiacol peroxidases because this phenolic compound has been extensively used as the colorimetric electron donor in the activity assays.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), discovered by Koukol and Conn (1961), is the entry enzyme between the shikimate pathway (primary metabolism) and the phenylpropanoid pathway (secondary metabolism) (Solecka 1997). PAL is mainly

located in the cytoplasm and catalyses a non-oxidative deamination of phenylalanine, coming from phosphoenol pyruvate via shikimic acid pathway, leading to the formation of trans-cinnamic acid (Petersen et al. 1999). In certain grasses and fungi, PAL also acts on tyrosine leading directly to the formation of 4-coumarate (Strack 1997).

PAL belongs to the class of carbon-nitrogen lyases that form a double bond, in contrast to dehydrogenation and hydrolysis. The active site contains a dehydroalanine residue whose methylene group binds to the amino group of the phenylalanine.

Hydroxyproline-rich glycoproteins (HRGPs) are the most abundant among the plant cell wall proteins. The HRGPs in the cell walls of plants are also termed as extensins, which was originally coined to suggest their role in cell wall extension (Lamport, 1967).

Structural proteins such as hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) are expressed or synthesised to restore the extracellular matrix after wounding (Bowles 1990). Hydroxyproline-rich glycoproteins (HRGPs) or extensins is a generic term that includes all molecules rich in hydroxyprolin and proline. They are involved in the control of cell wall structure and its strengthening by the formation of peroxidase mediated intermolecular cross-links in defence responses (Cooper and Varner 1984).

HRGPs may also play defensive roles as specific agglutinins of pathogens, developing structural barriers and providing sites for lignin deposition (Leach et al. 1982; Cassab and Varner 1988).

β-1,3-glucanases (EC 3.2.1.39) and chitinases (EC 3.2.1.14) catalyse the hydrolysis of β-D-glucosidic linkages in β-1,3-glucans and β-1,4-(2-acetammido-2-deoxy)-D-glucosidic linkages in chitin respectively (reviewed in Boller 1988). Both enzymes are constitutively expressed in different organs in higher plants and regulated by ethylene and other plant hormones. Additionally, these enzymes are induced in plant after pathogen attack and exposure to various biotic and abiotic stress (Boller et al. 1983, Simons et al. 1992). It was proposed that these inducible enzymes participate in the active defence of the plants to pathogens. They were identified among the pathogenesis-related (PR) proteins. β-1,3-glucanases are consider as one of the more important pathogenesis-related (PR) proteins (Dixon and Lamb 1990).

The study of the enzymatic activity of wounding related enzymes and of the ROS will also contribute to the understanding of the PPD process.

4.2 RESULTS

4.2.1 Samples management

Samples management was the same as described in chapter three.

4.2.2 Activity of phenylalanine ammonia lyase in response to PPD

Phenylalanine ammonia-lyase (PAL) activity, which was only determined in the June 1999 samples group, did not show a definable trend. A possible cause is the significant variability between replicates. Figure 4.1 shows PAL activity for low PPD (MBRA 12), medium PPD (MNGA 2) and high PPD (CM 2177-2) cultivars. Enzymatic activity in the low and high PPD cultivars did not show noticeable changes during the time course, while the medium cultivar showed a considerable increase in activity after 24 hours. This increase in activity may reflect the onset of wound responses. Then, after the sixth day there was another activity increase, but this time the increase was not so large as the one at the first stage of the deteriorating time course. The average of enzymatic activity of the three PPD levels showed the same profiles observed in figure 4.1. Cultivars of the medium PPD level showed their peak of activity one day after harvest, while low PPD cultivars presented the same frequency during the deterioration time course; and high PPD varieties showed their highest frequencies at days five and seven (appendix 8.4.2).

The REGWQ test did not differentiate the PPD level groups. Looking at cultivar differences, REGWQ test separated MNGA 2 from the rest at day one and seven. At the fifth day three groups were determined. The first formed by MNGA 2 and CM 2177-2, the second by MBRA 12 and the third comprised the remaining cultivars (appendix 8.3).

The results of PAL activity did not show expected trends, since the highest activity was not observed in a high PPD cultivar.

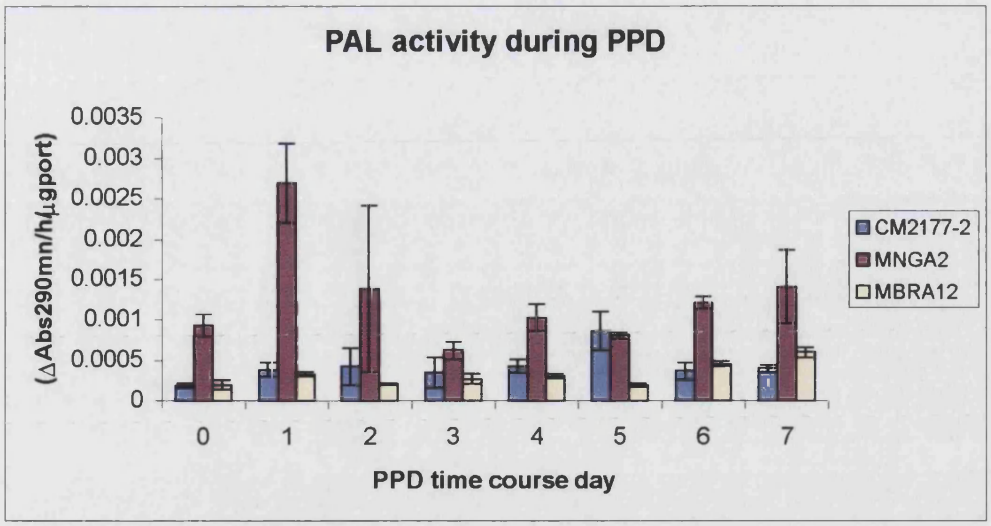


Figure 4.1 Phenylalanine ammonia lyase activity during PPD.

4.2.3 Activity of catalase in response to PPD

Catalase enzymatic activity, like PAL activity, showed differences among repetitions, which, again, made difficult the elucidation of a general trend. One example for catalase activity in varieties with different PPD susceptibilities is illustrated in figure 4.2 (samples of June 1999 group). The medium PPD cultivar showed the highest activity values compared to the others, particularly to the low PPD cultivar.

Catalase activity was also assayed with the December 1999 samples (Fig. 4.3). MNGA 2 also showed large values throughout the time course, but the difference from the CM 2177-2 values was not so marked as in the June 1999 data set. The activity of the low PPD cultivar, MBRA 337, was the lowest compared to the other cultivars. This result was equally observed in both data sets. The maximum activity for medium and high PPD cultivars in the June 1999 data group was observed at day four, while the maximum for the low PPD cultivar was detected at day six. This trend was similar to the one shown in the PPD level average activity graphs, but this trend did not totally coincide with the peak frequency. MNGA 2 showed both maximum activity and maximum peak frequency at the same time, while the low PPD cultivars peaked on the fifth day and the high PPD cultivars peaked on the first and third days. The latter observation was another demonstration of the significant variability between replications. The enzyme activity trend observed in December 1999 samples showed day two as the day for maximum activity in low and high PPD cultivars. Day two was

also observed to present the maximum values in the average PPD level graphs (appendix 8.4). MNGA 2 showed the highest activity at day four, but it was not possible to compare this observation with the results presented in the PPD levels average activity graphs, since no difference in activity was observed between day one to day four.

The REGWQ test did not separate groups by PPD level for either sampling season. At cultivar level the formation of groups was observed. The June 1999 samples showed group separations at days 0, 4, 5 and 7. The common trait among those groups was the presence of MNGA 2 in the group with the larger activity mean value. Looking at the area under the curve, MNGA 2 was also separated from the rest of cultivars. The December 1999 samples showed groups separation at days 1, 2 and 4. Like in the June 1999 season, MNGA 2 showed the higher activity mean values. The REGWQ test by area under the curve, also showed MNGA 2 separated from the rest of cultivars (appendix 8.3).

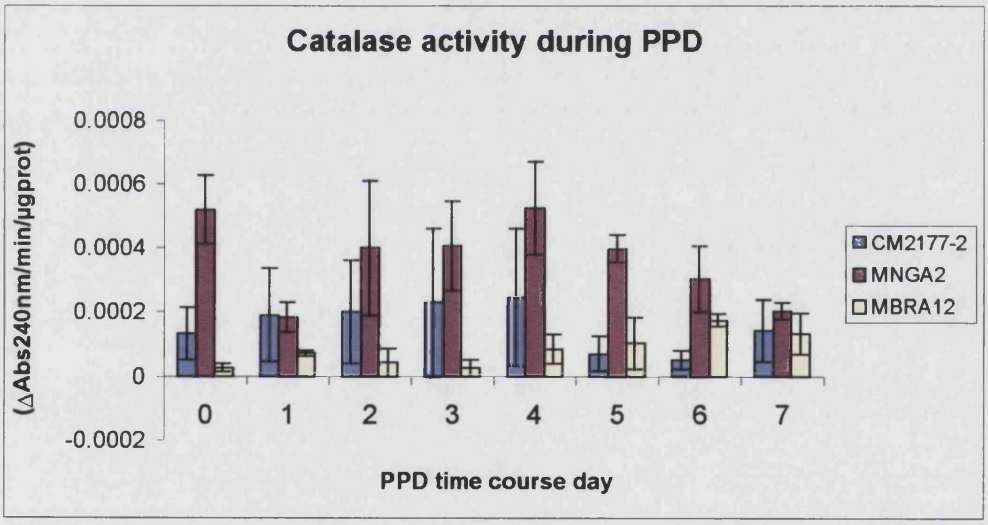


Figure 4.2 Catalase activity during PPD (June 1999 sampling).

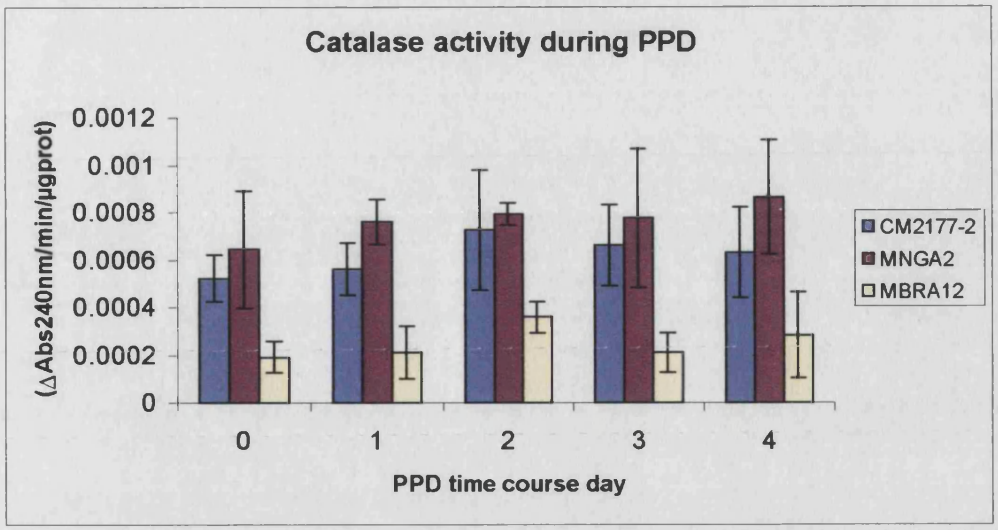


Figure 4.3 Catalase activity during PPD (December 1999 sampling).

4.2.4 Activity of peroxidase in response to PPD

POX activity was measured in all data groups. The general trend observed in the June 1999 sampling group is the increasing of activity during the time course (Fig. 4.4). High PPD cultivars showed the larger activity values, as might be expected knowing POX has been positively associated with PPD (Campos and Carvalho 1990). Cultivars also differed in the time when the activity increase became most evident; day six for low PPD cultivars, day four for medium PPD cultivars and day five for high PPD cultivars. High PPD cultivar (CM 2177-2) and medium PPD cultivar (MNGA 2) showed a slight activity increase at day one, but this increase was not comparable with the one observed between days four and five. The earlier increase in activity might correlate with the onset of vascular streaking. When cultivars were grouped by PPD susceptibility (see appendix 8.4) observations were very similar to the example in figure 4.4. Higher activity values appeared by the end of the storage time course. This observation was strengthened by the larger peak frequencies at days six and seven for all PPD levels. Though the enzyme activity graphs showed some differences, statistical analysis did not determined significant differences between PPD levels. Looking at differences between cultivars, CM 2177-2 separated from the rest from day one. After day four MNGA 2 and CM 2177-2 constituted one group, which separated from the rest. But, by the last day all cultivars formed one group.

POX activity calculated in the December 1999 data set showed different trends. The medium PPD cultivar showed the highest values for activity, while in June 1999 larger values were present in the high PPD cultivar. In the December 1999 samples, the slight increase in activity observed at day two in the June 1999 samples became more evident. These observations correlate better with the onset of PPD. A difference in time for activity increase was also observed. The time for the activity peak in high and medium PPD cultivars was day one, while for the low PPD cultivar the peak occurred at day two. The graph showing the average activity value for the different PPD levels (see appendix 8.4) did not fully reflect the trend observed in figure 4.5. High PPD level cultivars peaked at day 2 while CM 2177-2, a high PPD level cultivar, peaked at day one. The days for maximum activity by PPD level concurred in the same day when higher peak frequency was observed.

The REGWQ test did not show significant differences between PPD level groups. For all cultivars, the REGWQ test showed different groups during the time course, except on day two (see appendix 8.3). The number of groups varied throughout the time course. Three groups were separated at day zero, four groups at day one, five groups at day three and nine at day four. It was observed that during the time course MPER 183 showed the larger mean and MBRA 12 showed the lower means activity values.

Family K data group did not show significant differences between the PPD level groups. Activity values for low PPD cultivars were almost the same between days one and four. Medium PPD level cultivars showed higher values at days one and two, and high PPD cultivars on days one and three. Higher frequency peaks were observed at day three for low and high levels and day one for medium level (appendix 8.4.4). The REGWQ test separated low PPD cultivars from the other two groups at day zero, and at days three and four high PPD level cultivars were differentiated from medium and low level cultivars (appendix8.3.1.4).

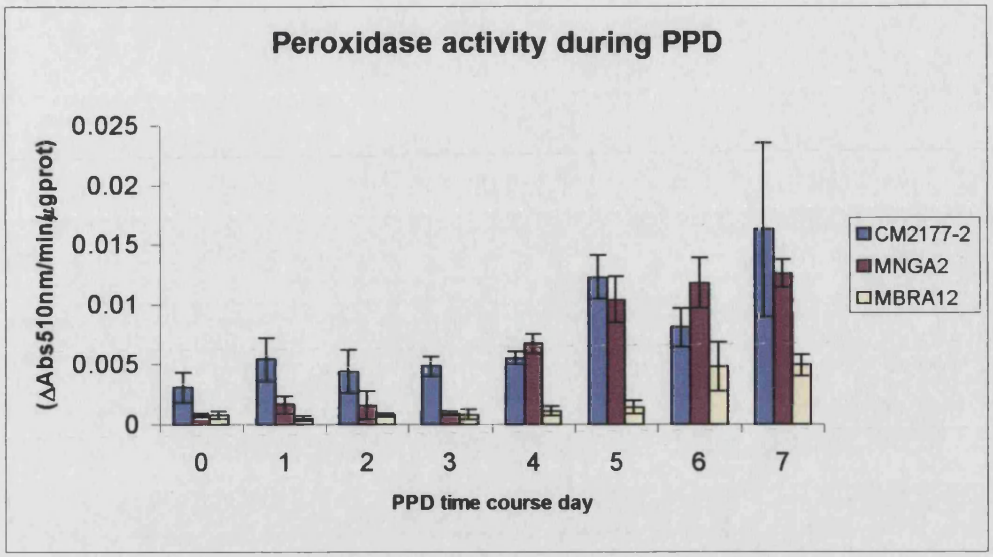


Figure 4.4 Peroxidase activity during PPD (June 1999 sampling).

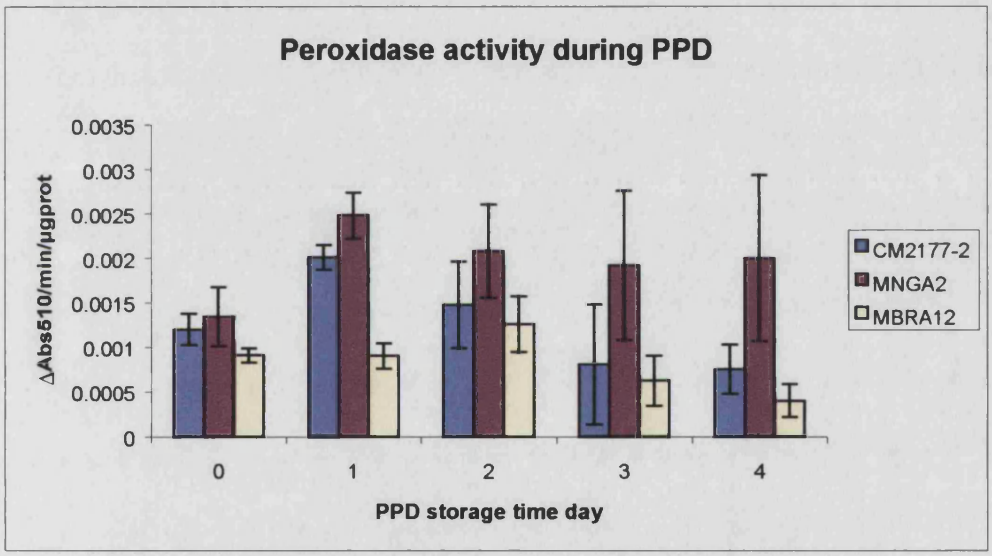


Figure 4.5 Peroxidase activity during PPD (December 1999 sampling).

4.2.4.1 Detection of peroxidase isoforms by isoelectro focusing in polyacrylamide gel electrophoresis

The detection of cassava root peroxidase (POX) isoforms was carried out in cultivars CM 523-7 and MPER 183, high and low PPD respectively, in a post harvest time course of four days (Fig. 4.6). One cationic POX isoform (pI: 8.8) was detected in both cultivars throughout the time course. The detected anionic POX isoforms showed differences between cultivars. Only one anionic isoform (pI 3.3, appearing immediately

after harvesting) was common for both cultivars. This isoform, as with the anionic one mentioned previously, did not show differences in intensity during the time course. There were two other isoforms at the same pI (5.68 and 4.87) for both cultivars, but they showed differences during the time course. The isoform at pI 5.68 was present in MPER 183 from harvesting, but it increased considerably in intensity from day two. In CM 523-7 this isoform was only detectable from day two, and, as in MPER 183, the isoform intensified with time. The pI 4.87 isoform occurred in CM 523-7 throughout the time course, but in MPER 183 it was only detectable from day one. This isoform intensified with time in both cultivars, but the activity in CM 523-7 was notably higher than MPER 183. An isoform with pI 4.61 was only present in CM 523-7, occurring immediately after harvesting and increasing in intensity with time. There was another isoform (pI 5.32) present only in CM 523-7. Bearing in mind that peroxidases have been associated with PPD, this isoform may be a point of interest for its detection in a high PPD cultivar, its occurrence from 48 hours after harvesting and its noticeable increase of activity along the time course. This isoform might be a good marker associated with high PPD varieties.

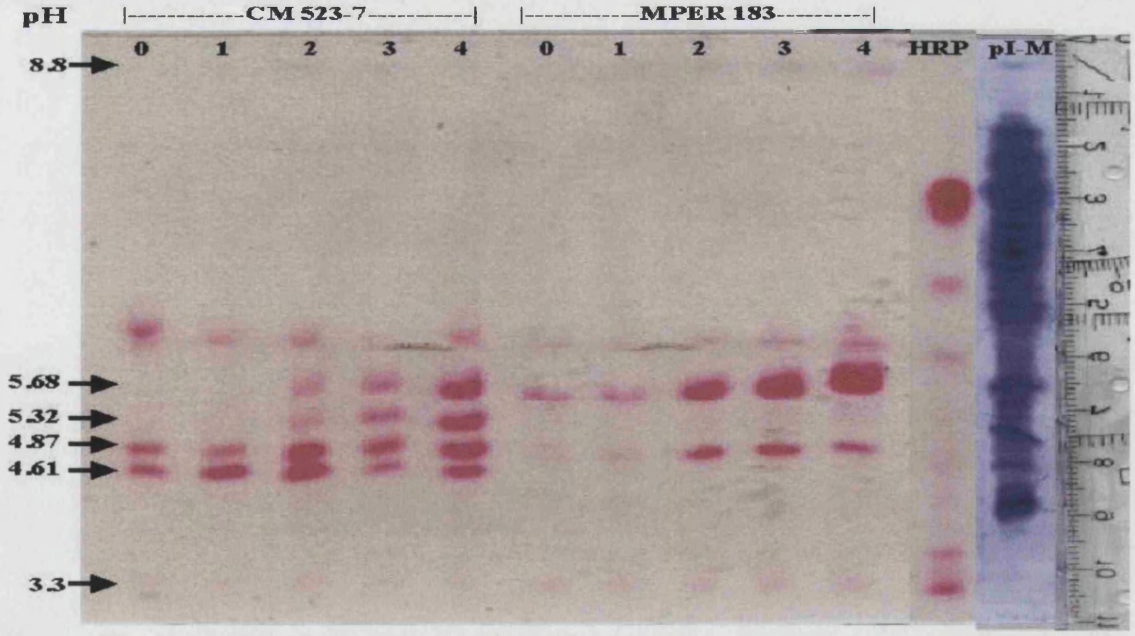


Figure 4.6 Detection of peroxidase isoforms by isoelectro focusing in polyacrylamide gel electrophoresis in cultivars CM523-7 (high PPD) and MPER 183 (low PPD), during a post harvest time course of four days. Isoforms were revealed by enzymatic activity reaction with 4-aminoantipyrine and 3,5-dicholoro-2-hydroxy benzene sulphonic acid. HRP: horse radish peroxidase, used as positive control. pI-M: isoelectric point marker stained with Coomasie blue R.

4.2.4.2 Peroxidase activity localisation by tissue printing

POX localisation in cassava roots by nitrocellulose tissue prints was visualised by enzymatic activity reaction with 4-aminoantipyrine (AA) and 3,5-dicholoro-2-hydroxy benzene sulphonic acid (DHBS). High PPD (CM 2177-2 and CM 523-7), medium PPD (MNGA 2 and MVEN 77) and low PPD (MBRA 337, MBRA 12 and MPER 183) cultivars were included in this assay, following a time course of four days (Fig. 4.7). Tissue prints showed that immediately after harvesting POX activity was concentrated at the inner cork layer of the root. Following the course of deterioration, POX activity spread first to the xylem parenchyma and then to the storage parenchyma including the primary xylem. The best example of spreading of the peroxidase activity is observed in CM 523-7. It was interesting that the intensification of POX-derived coloration paralleled the development of the vascular streaking and browning progress. In the lowest PPD cultivar (MBRA 337), the POX activity was mostly concentrated in the inner cork and the primary xylem.

POX activity was also detected by enzymatic activity with guaiacol (Fig. 4.8). Oxidation of guaiacol results in a dark brown precipitate which is easier to detect compared with the reddish precipitate product of the oxidation of 4-aminoantipyrine and 3,5-dicholoro-2-hydroxy benzene sulphonic acid. Observations were basically the same as the experiment with AA and DHBS. The exception was that immediately after harvesting POX activity could be detected in the xylem vessels.

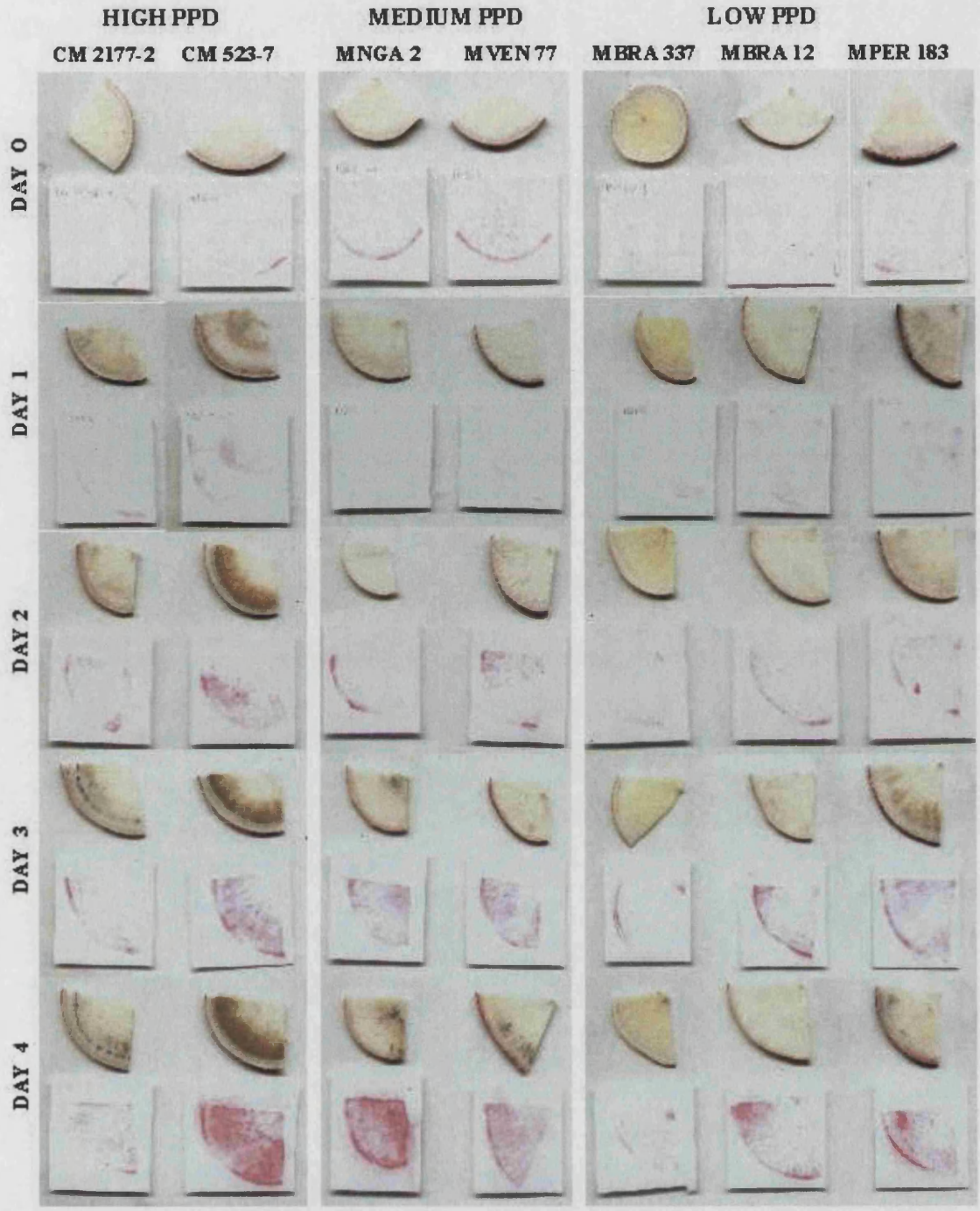


Figure 4.7 Localisation of peroxidase activity by tissue printing on nitrocellulose membranes. Enzymatic activity was visualised with 4-aminoantipyrine and 3,5-dicholoro-2-hydroxy benzene sulphonic acid.

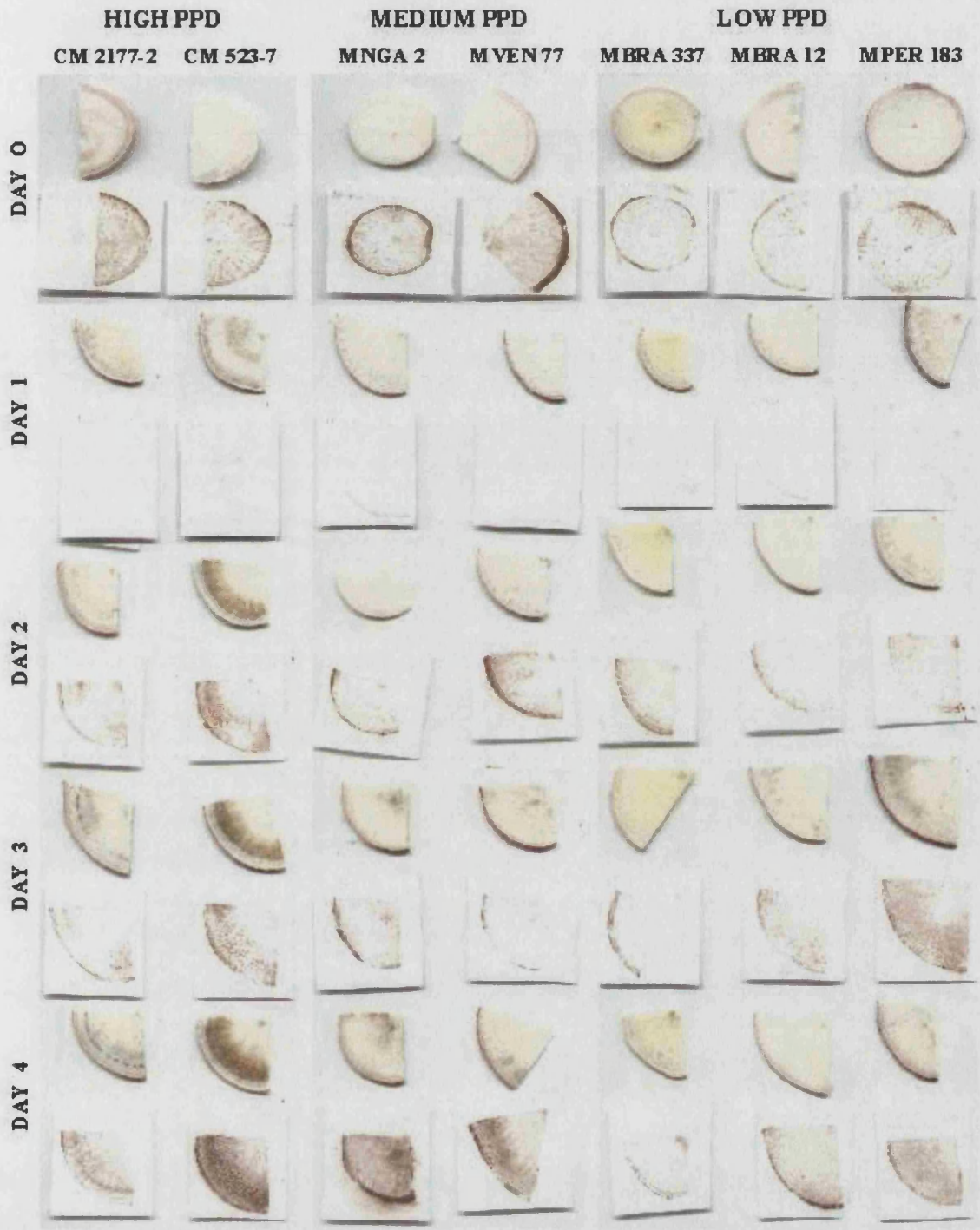


Figure 4.8 Localisation of peroxidase activity by tissue printing on nitrocellulose membranes. Enzymatic activity was visualised with guaiacol.

4.2.5 Activity of polyphenol oxidase in response to PPD

PPO was assayed in all groups of samples, showing the most interesting trend in the June 1999 group. Figure 4.9 shows the data for cultivars with different susceptibilities towards PPD, CM 2177-2 (high), MNGA 2 (medium) and MPER 183 (low). PPO activity of MNGA 2 and MPER 183 showed no remarkable differences during the time course, while CM 2177-2 showed a significant activity increase after day five. The same trend was observed in the average level activity graph (appendix 8.4). This general trend, of positive increasing of activity with the progress of PPD in susceptible cultivars, was confirmed by the observation of higher peak frequency at day seven. The REGWQ test did not show significant differences between PPD level groups, but considering all cultivars, results showed the separation of CM 2177-2 from the other cultivars from day one.

PPO activity in the December 1999 samples showed the general trend of increasing activity during the time course, but in contrast to observations in June 1999, high and low PPD cultivars showed very similar values. Peaks frequency concurred with the observation of maximum activity at day four (appendix 8.3). The REGWQ test did not determine significant differences between levels, but by cultivar the test showed different groups from day one. In all groups, cultivar MPER 183s showed the higher mean values.

Family K samples showed again the trend of parallel increase of activity with PPD progress. But, higher values were present in cultivars with low and medium PPD level (appendix 8.4). However, the REGWQ test did not separate groups by means of PPD level groups.

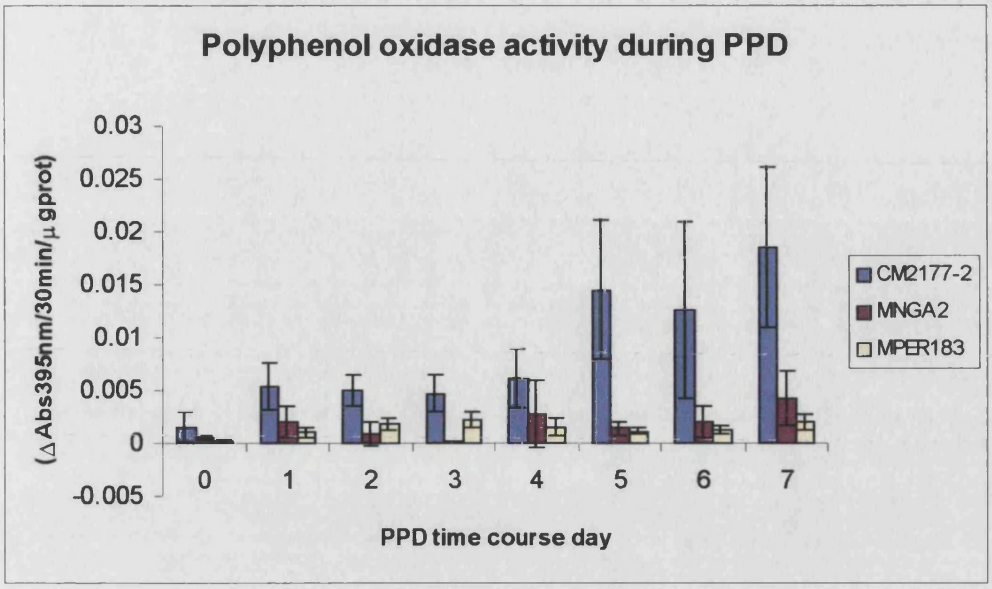


Figure 4.9 Polyphenol oxidase activity during PPD (June 1999 data group).

4.2.5.1 Polyphenol oxidase activity localisation by tissue printing

PPO activity localisation was carried out as previously described for peroxidases. PPO activity was visualised by the oxidation of (+)-catechin, which in its oxidised form turns to a light ochre precipitate. Tissue prints are presented in figure 4.10. Localisation of PPO activity was limited to the cortical parenchyma, and no marked differences were observed between cultivars. Only MPER 183 showed a stronger staining compared with the rest of cultivars, but no difference in colouration intensity was detected over the time course.

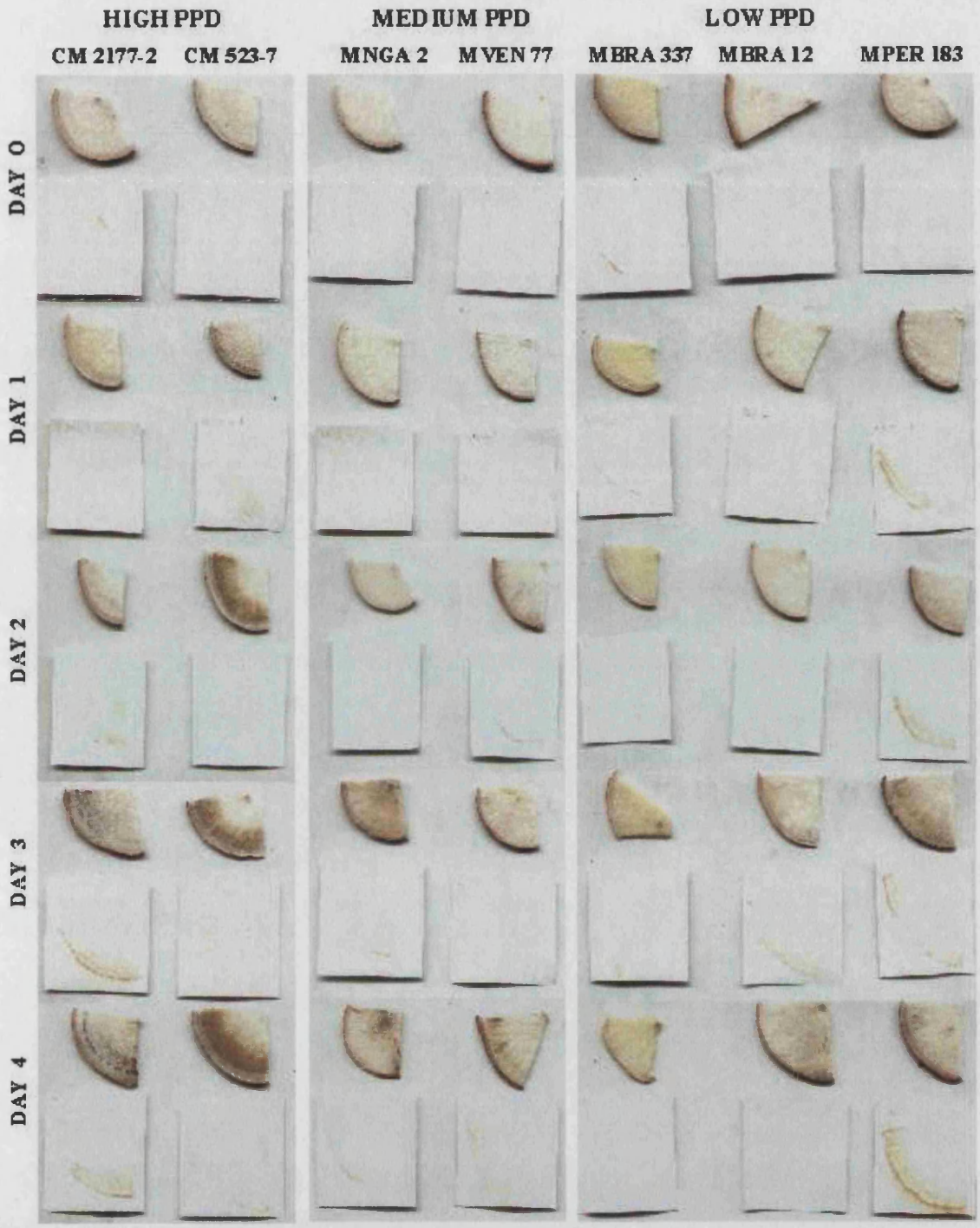


Figure 4.10 Localisation of polyphenol oxidase activity by tissue printing on nitrocellulose membranes. Enzyme activity was visualised by oxidation of (+)-catechin.

4.2.6 Activity of β-1,3-glucanase in response to PPD

β-1,3-glucanase activity showed a general tendency of increasing activity with time. Figure 4.11 shows the enzyme activity in cultivars with contrasting responses towards PPD in a time course of seven days. During the first four days there were not noticeable differences between cultivars. After day five differences became more evident, the high PPD cultivar (CM 2177-2) having the larger activity. The same pattern was observed in PPD level enzyme activity graph. The frequency peak graph also reflected previous observations. The accumulation of enzyme activity occurred at day seven for medium and high PPD varieties. Low PPD varieties showed the same peak frequency during the last PPD time course days. The REGWQ test did not find significant differences between PPD level groups over the time course of deterioration, but there was a separation of CM21777-2 from the rest of the group at day seven. Looking at the area under the curve, three groups were determined. As expected, CM 2177-2 constituted the group with the largest activity. The third group in activity order corresponded to MPER 183, low PPD; and the second group comprised the remaining cultivars.

The increased activity timing did not suggest an association between β-1,3-glucanase activity and physiological deterioration. It might reflect an association with microbial deterioration, since secondary deterioration occurred four to five days after harvesting.

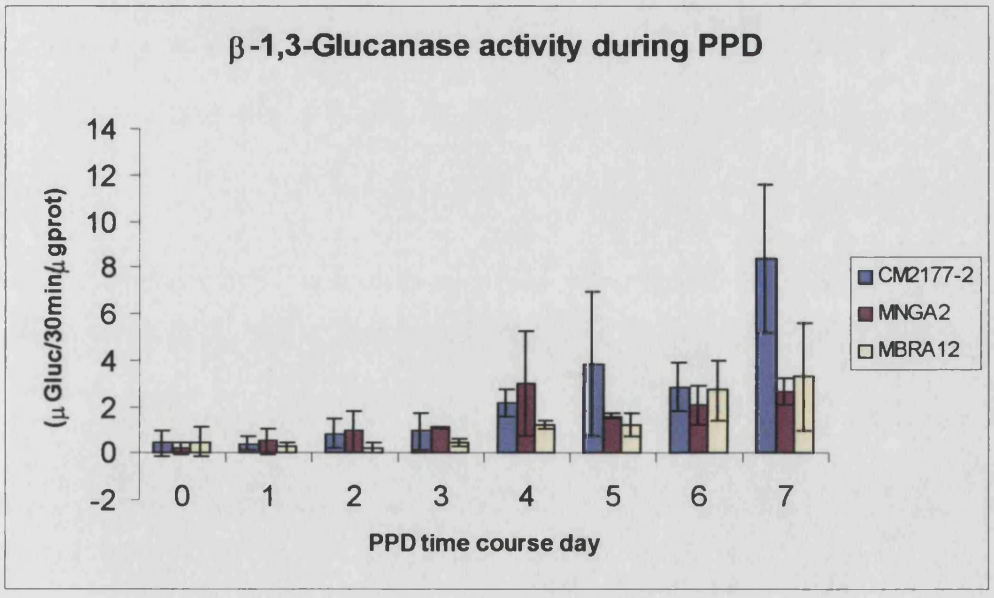


Figure 4.11 β-1,3-glucanase activity during PPD.

4.2.7 Activity of chitinase in response to PPD

Trends in chitinase activity were very difficult to determine due to the high variations between replicates. Figure 4.12 shows profiles for high, medium and low PPD cultivars. The activity of the low PPD cultivar, MPER 183, did not show visible changes along the time course. MNGA 2, medium PPD, showed higher values during the first three days. CM 2177-2 showed a similar pattern to MNGA 2 until day four, but then the activity increased dramatically at days five and seven. The large standard errors between samples at day 5 and 7, and the low activity at day 6 did not permit the detection of a possible trend with any confidence.

PPD level enzyme activity graphs exhibited the same features as figure 4.12 In the frequency peak graphs no well-defined peaks were observed. As it was generally observed, no different groups at PPD level were determined by the REGWQ test. By means of cultivar, the REGWQ test separated CM 2177-2 from the other cultivars from day four to day seven. Considering the area under the curve, CM 2177-2 was also separated from the other cultivars.

As it was suggested for β-1,3–glucanase, chitinase could be associated with microbial deterioration. It is known for its role as a pathogen defence enzyme in other fully studied plant systems (Dixon and Lamb 1990).

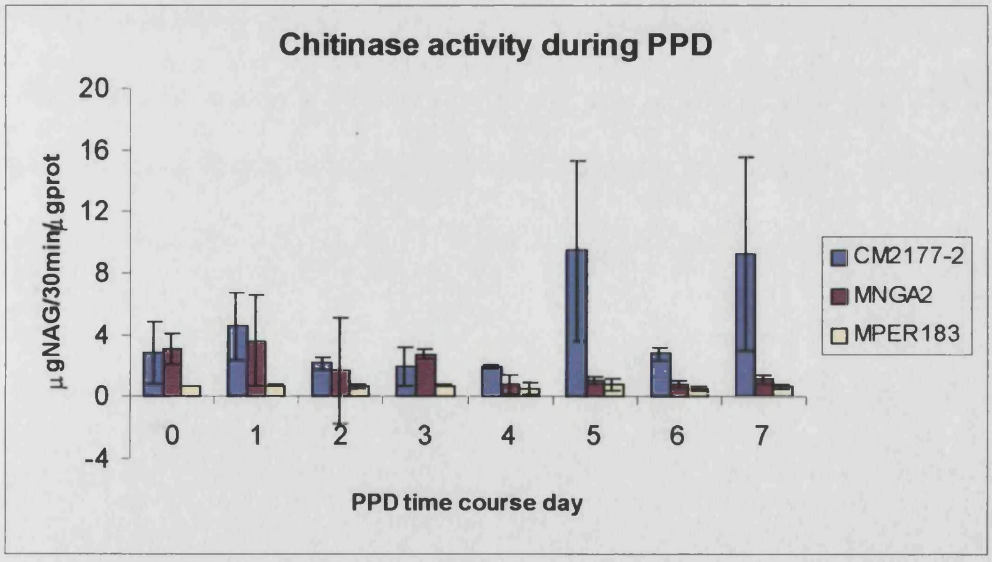


Figure 4.12 Chitinase activity during PPD.

4.2.8 Activity of scopoletin-peroxidase enzyme during PPD.

Based on the involvement of hydroxycoumarins and peroxidase enzyme activity in PPD, coumarins were tested as phenolic substrates for deteriorated cassava root enzymatic extracts. Assays were made using scopletin, esculin and esculetin as phenolic substrates. An enzymatic extract of two days harvested CM 2177-2 was used as source of cassava peroxidase. Positive enzymatic reaction controls were made by using guaiacol, a well known POX phenolic substrate, commercial purified peroxidase (horse radish peroxidase, HRP) and negative controls were tested by exclusion of H_2O_2 from the reaction mixture. Figure 4.13 summarises the reaction mixture combinations with the different phenolic subtracts and the resulting colouration reaction. All hydroxycoumarins reacted positively by the H_2O_2 mediated oxidation. The most interesting reaction was shown by scopoletin. Immediately after addition of H_2O_2 to the reaction solution containing peroxidase extract and scopoletin, the solution turned to a dark blue colour. After approximately 5 min the solution started to turn green and then slowly paled until a yellowish colouration. Additionally and most remarkably, the formation of a dark blue-black precipitate was observed at the same time as the reaction mixture paling. This reaction may explain the decrease of scopoletin and H_2O_2 (Buschmann et al. 2000) after two to three days of harvesting; and sheds some light on vascular streaking explanation. Esculetin and esculin reaction turned light brown in colouration as the guaiacol reaction, but no dark precipitate was observed.

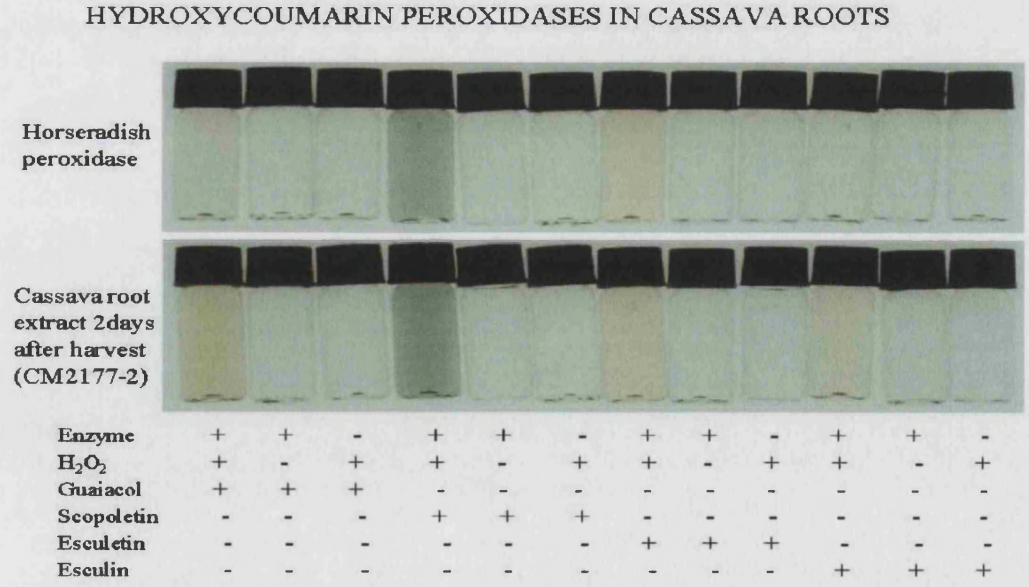


Figure 4.13 Hydroxycoumarin peroxidases in cassava root enzymatic extracts.

Quantification of the scopoletin peroxidase activity in cultivars with different responses towards PPD is shown in figure 4.14. As had been observed in all enzyme activity quantifications, high variations between replicas were detected, but it is possible to observe a general trend of increased activity during the time course of PPD progress. As well, the higher values in activity for the susceptible cultivar (CM2177-2), particularly from day two, were observed. Like the three cultivars plotted in figure 4.14, the general tendency of the December 1999 group cultivars was the increase of activity over the PPD time course, having all PPD levels their maximum average activity at day four (appendix 8.4.3). The medium PPD level cultivars showed higher activities during the first three days, but then high PPD cultivars exceeded medium PPD cultivars. Frequency peak graphs for low and high PPD cultivars only showed two peaks at day three and four, being the larger at day four. Medium PPD level cultivars showed peaks after harvesting with their maximum at day four. However, the REGWQ grouping analysis did not separate the samples by PPD levels. There was only a significant separation by cultivars at day four. MVEN 77 separated as one group, presenting the highest mean value. SM 985-9, CM 2177-7 and MDOM 5, the most PPD susceptible cultivars, formed the second group. The third group consisted of two low PPD cultivars, MBRA 337 and MPER 183. The last group was a mix of high, medium and low PPD varieties.

The Family K sampling group showed the same tendencies observed in the December 1999 group. Maximum values of activity were observed at day four. High PPD genotypes showed the larger mean values, but the differences with the other levels was not very significant. The peak frequency graph showed the presence of peaks from day two, but at a very low frequency compared with the larger frequency at day four (appendix 8.4.4). The REGWQ grouping only separated PPD levels at day three, low level separated from the rest.

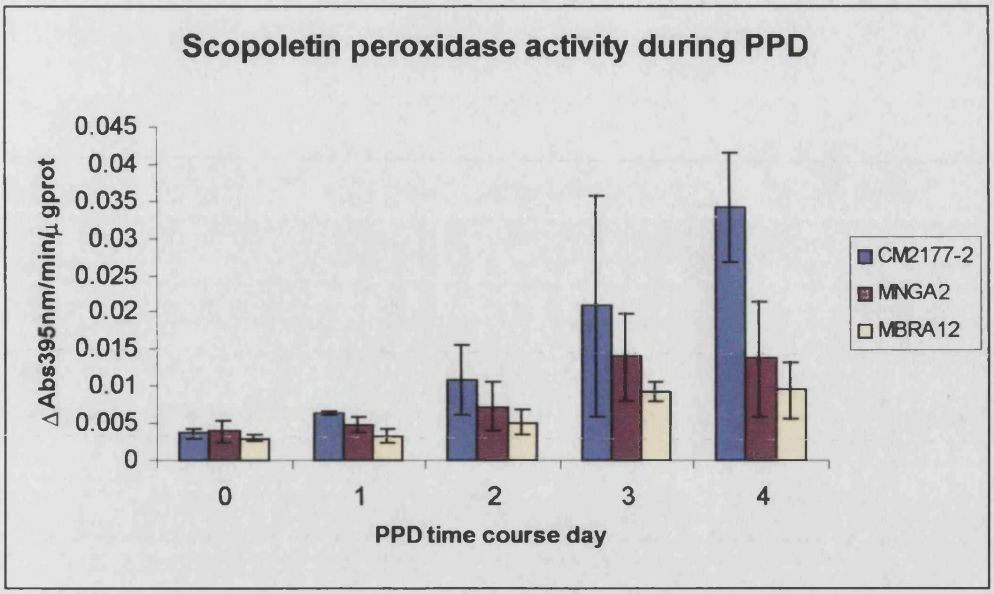


Figure 4.14 Scopoletin-peroxisase activity during PPD.

4.2.8.1 Detection of scopoletin-peroxidase isoforms by isoelectro focusing in polyacrylamide gel electrophoresis

The detection of scopoletin-peroxidase (SCP-POX) isoforms was carried out in cultivars CM 523-7 and MPER 183, high and low PPD respectively, following a post harvest time course of four days (Fig. 4.15). Cationic isoforms were not detected, while six anionic isoforms were visualised. Close to the anode baseline a diffuse band at pH 3.3 was observed. It occurred with the same intensity along the time course in CM 523-7, whereas in MPER 183 its intensity seemed to increase. Because the colouration of the band was quite faint, it was not possible to observe if the diffuse band corresponded to just one isoform or more than one highly anionic peroxidases. Besides this observation, the detection of only anionic scopoletin-peroxidases also suggested the use of narrower pH range IEF gels to obtain a better separation of these isoenzymes. Isoforms with pI 5.58 and 5.13 were present in both cultivars and increased in intensity with time. There was another isoform common for both cultivars, pI 4.61, but it appeared in CM 523-7 after day two. Cultivar MPER 183 showed two more isoforms (pI 4.74 and 4.48). Both isoforms were present throughout the time course, but an increase in activity with time was only visible in 4.74 pI isoform.

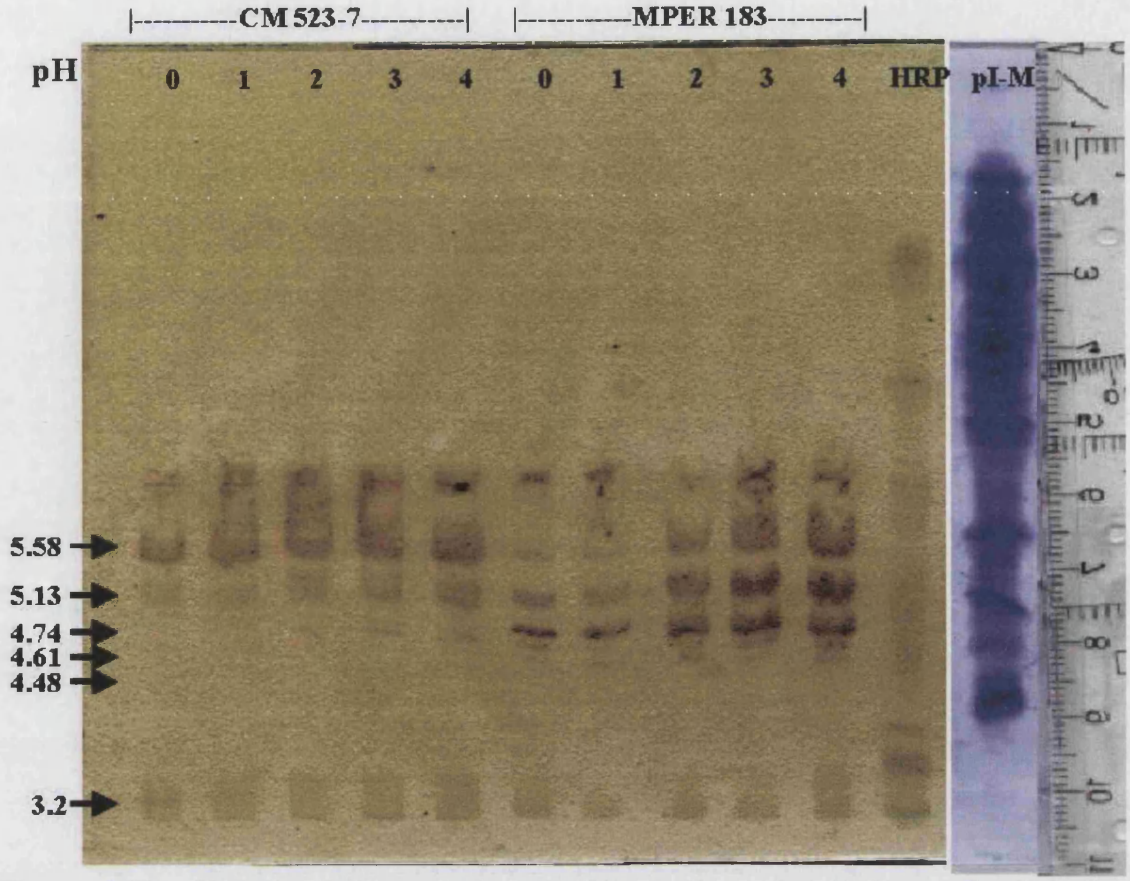


Figure 4.15 Detection of scopoletin-peroxidase isoforms by isoelectro focusing in polyacrylamide gel electrophoresis in cultivars CM523-7 (high PPD) and MPER 183 (low PPD), during a post-harvest time course of four days. Isoforms were revealed by enzymatic activity reaction with scopoletin. HRP: horse radish peroxidase, used as positive control. pI-M: isoelectric point marker stained with Coomasie blue R.

4.2.8.2 Scopoletin peroxidase activity localisation by tissue printing

SCP-POX activity localisation in cassava roots undergoing PPD, during a time course of four days, was performed as POX tissue printing in high PPD (CM 2177-2 and CM 523-7), medium PPD (MNGA 2 and MVEN 77) and low PPD (MBRA 337, MBRA 12 and MPER 183) cultivars. Tissue prints are shown in figure 4.16. Localisation of SCP-POX followed the same pattern observed in tissues prints for POX (section 4.2.4.2). However, the spreading of SCP-POX activity to the storage parenchyma was notably faster. This SCP-POX spreading was so prominent for all cultivars, that by the fourth time course day it was not possible to detect differences between cultivars with contrasting responses to PPD.

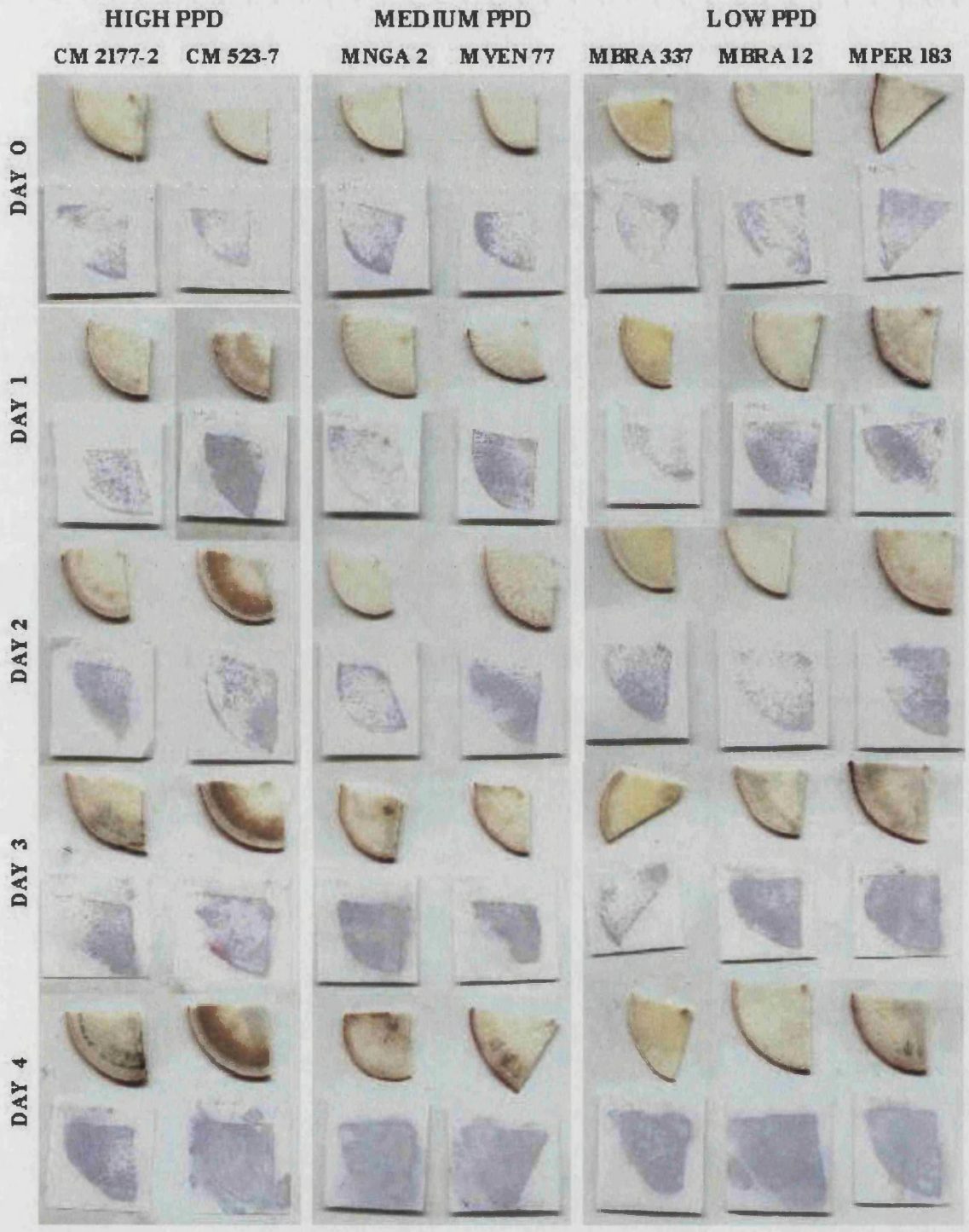


Figure 4.16 Localisation of scopoletin-peroxidase activity by tissue printing on nitrocellulose membranes.

4.2.9 Hydroxyproline rich glycoproteins localisation by tissue printing

Studies on the occurrence of wound healing process elements in cassava roots may help to shed light on the suggestion that this repair process is inadequate in harvested cassava tuberous roots. Detection of HRGPs or extensins was performed by probing nitrocellulose tissue prints with a polyclonal antibody, obtained from a purified carrot extensin. Low PPD (MPER 183) and high PPD (CM 523-7) cultivars were assayed following a post harvest time course of four days (Fig 4.17). Tissue prints showed that HRGPs accumulated all over the root tissue, particularly in the vascular parenchyma, during the deterioration time course. In addition, a parallel increase in colouration intensity with the progress of deterioration was observed. This intensity accumulation was more pronounced in the high PPD cultivar.

4.2.9.1 Confirmation of HRGPs presence in cassava roots enzymatic extracts.

A Western blot with soluble HRGPs was assayed with the purpose of eliminating possible artefacts in the detection of HRGPs by tissue printing. Following the same procedure used with tissue prints, root protein extracts from cultivars CM 523-7 and MPER 183 undergoing PDD over a time course of four days were tested (Fig. 4.18). The molecular weight of extensins was calculated by means of prestained SDS-page standards low range from Bio-Rad (catalog #161-0305), using the SeqAid II (version 3.60) programme. Extensins with molecular weights between 80500 and 43200 Da were common to both cultivars. Differences in intensity throughout the time course were not observed. The high intensity observed in MPER 183 immediately after harvesting and at first day is an artefact of the picture. CM 523-7, showed three faint additional bands. A band of 35422 Da molecular weight occurred throughout the time course. As with the other bands, its intensity remained constant during the time course. The second band, 31097 Da, was only detectable in the second day of the time course. While it is difficult to visualise in the picture, the third band, 25414 Da, appeared after two days of harvesting. The occurrence of the additional bands in cultivar CM 523-7 may explain the wide spread presence of extensins all over the root tissue compared with MPER 183.

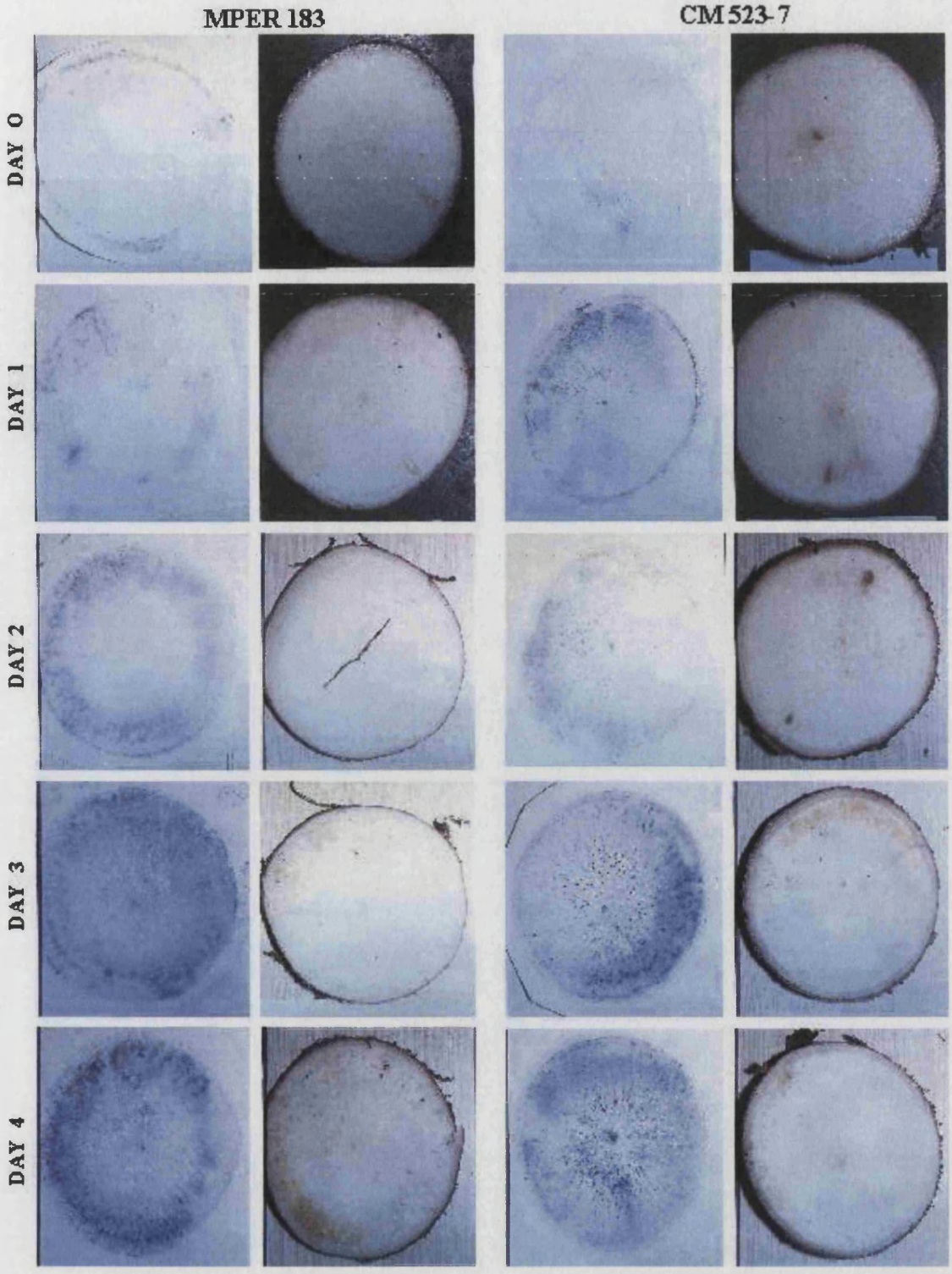


Figure 4.17 Localisation of HRGPs by detection with anti-HRGP antibody in on nitrocellulose tissue prints of cultivars MPER 183 and CM 523-7 undergoing PPD.

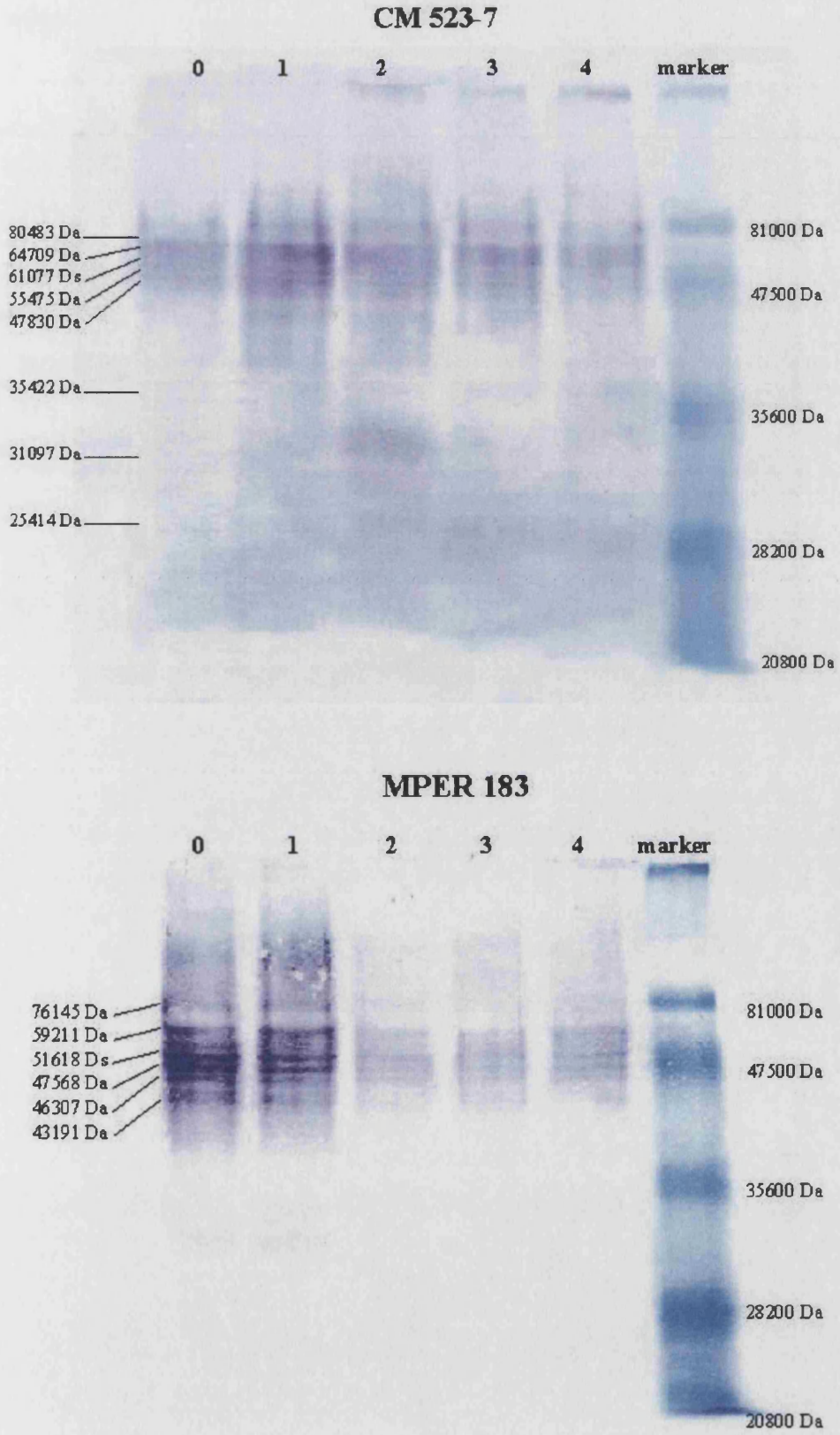


Figure 4.18 Western blot for soluble HRGPs in cassava root tissues undergoing PPD. HRGPs were detected by probing with anti-HRGP antibody isolated from carrots.

4.2.10 Localisation of reactive oxygen species (H_2O_2) in deteriorating cassava roots.

Hydrogen peroxide localisation, *in situ*, was made by vacuum infiltration with 3,3 diaminobenzidine tetrahydrochloride (DAB) followed by incubation for 3 h. Detection of H_2O_2 is visualised as a brown precipitate formation. Control reactions were made by co-infiltration of DAB and ascorbic acid (H_2O_2 scavenger). Results shown in figure 4.19 indicate that H_2O_2 accumulation occurs at the vascular parenchyma at first stage of vascular streaking. Then the H_2O_2 spread to the storage parenchyma. Differences between cultivars were observed. H_2O_2 detection in medium and low susceptible cultivars was barely visualised, while in high susceptible cultivars the accumulation was very evident after one day of harvesting. Cultivar MPER 183 was the exception compared to other low PPD cultivars, H_2O_2 accumulation was easily detectable from day two.

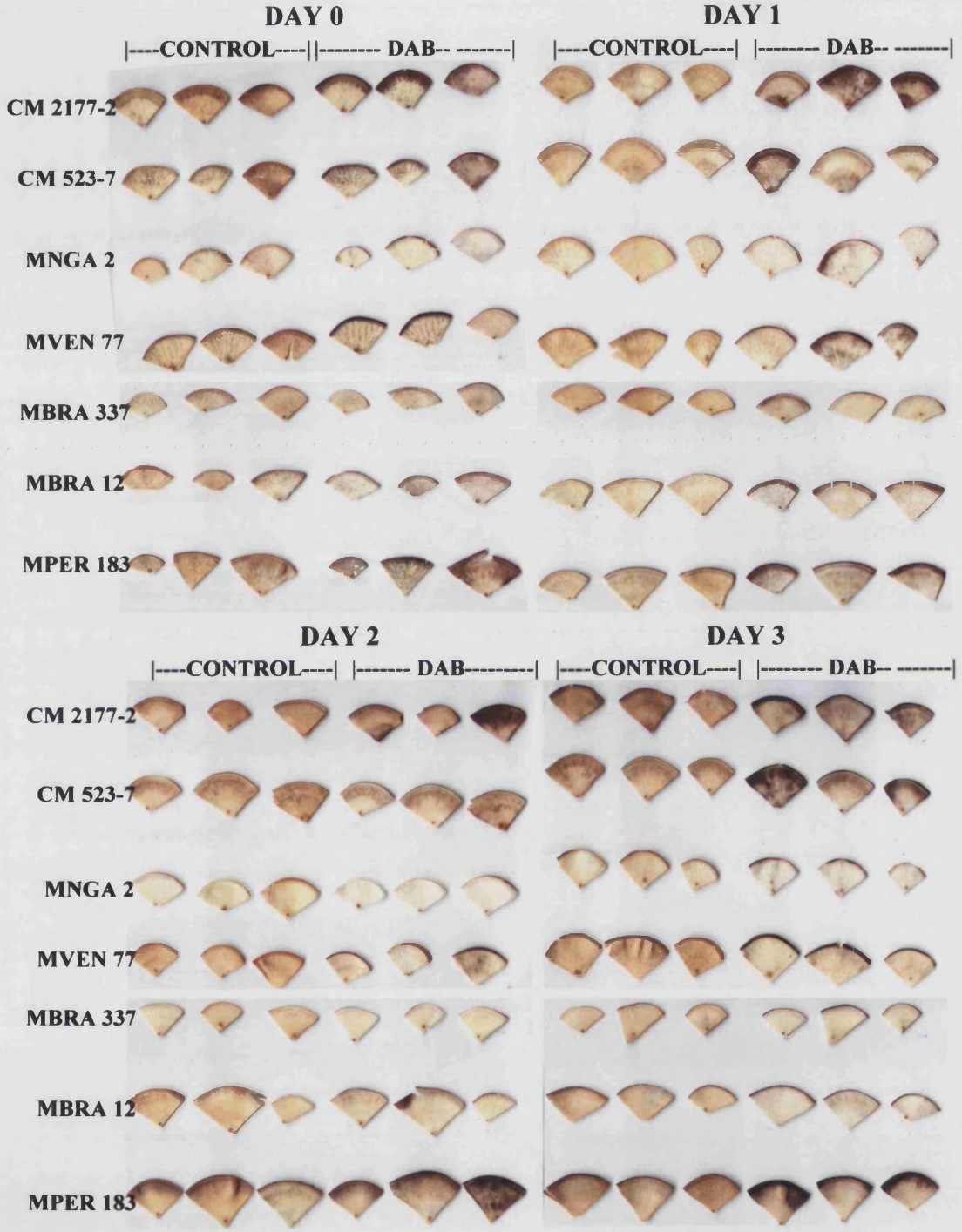


Figure 4.19 Detection, *in situ*, of H_2O_2 production in cassava roots during PPD. Detection was made by vacuum infiltration of DAB in root slices of high PPD (CM 2177-2 and CM 523-7), medium PPD (MNGA 2 and MVEN 77) and low PPD (MBRA 337, MBRA 12 and MPER 183) cultivars. Controls were co-infiltrated with ascorbic acid, a H_2O_2 scavenger.

4.2.11 Principal Component Analysis

The principal component analysis (PCA) was conducted in order to study the separation of cultivars during the PPD time course considering all enzymes in the analysis. We wanted to determine the key enzymes of the PPD response, i.e. the enzymes determining the separation of the cultivars in PPD-levels. However, this could not be achieved because clusters identified through the analysis were composed of a mixture of cultivars from different PPD levels.

The results of the PCA analysis are shown in appendix 8.6.2 and in Figure 4.20. Figure 4.20 shows three-dimensional graphs showing the relationships of cassava cultivars after enzymatic activities measurements.

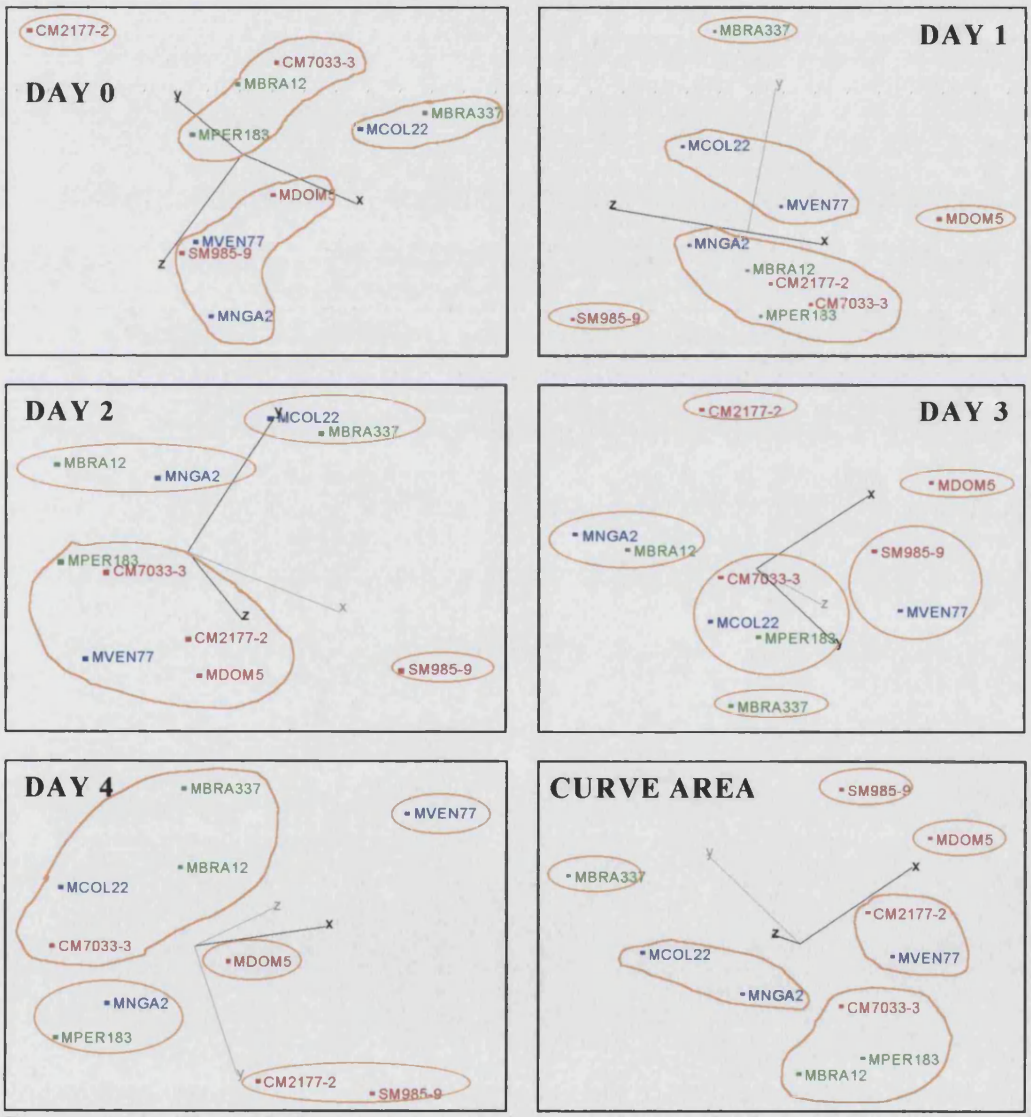


Figure 4.20. Three-dimensional graph from a Principal Component Analysis showing relationships of cassava cultivars after enzymatic activities measurements day by day and area under the curve.

4.2.12 Correlation analysis

A correlation analysis was performed, for each group of samples, among measurements of the different secondary metabolites, among enzymatic activity for all enzymes and between measurements of secondary metabolites and enzymatic activities. The correlations were calculated comparing the data generated day by day during the time course and comparing the AUTC in all cultivars and separating the cultivars by PPD levels. All results for the correlation analysis are presented in appendix 8.5. The first observation to mention is that the results of the correlation calculated by the comparison of the data day by day did not show the same correlations when considering the AUTC. The most important and informative correlations will be highlighted for the day by day measurements. In the June 1999 group samples, a positive correlation was shown between PPO and POX for all PPD levels and for all cultivars (correlation coefficients appear in section 8.5). Also, scopoletin and PPD-marker and peroxidase and PPD marker showed a high positive correlation for the high PPD-level cultivars. The high correlation between scopoletin and scopolin was clear for all cultivars and the three PPD levels.

In the December 1999 group of samples, the most important correlations were observed between SCP-POX and esculin, SCP-POX and PPD-marker and between PPO and PPD-marker and esculin and PPD-marker. All correlations were highly significant for all PPD levels and for all cultivars. Again, as shown in the June 1999 group of samples, scopolin and scopoletin showed significant correlations in all PPD levels and among all cultivars, suggesting as previously shown that the two metabolites increase in their concentration over the time course.

Finally, in the Family K group of samples, scopolin and scopoletin were highly correlated as shown in the two previous group of samples. Other significant and high correlations were observed for PPD-marker and scopoletin, PPD-marker and SCP-POX, PPD-marker and scopolin, and PPD-marker and esculin. All these correlations were significant and high for all PPD-levels and among all cultivars.

In this group of samples, SCP-POX showed a high correlation with several secondary metabolites: scopoletin, scopolin, and esculin for all cultivars and for all PPD-levels.

Peroxidases, in cassava roots, catalyse a reaction between scopoletin and H_2O_2 resulting in a black precipitate. The localisation of POX activity around the vascular parenchyma suggests a correlation between PPD and oxidation of hydroxycoumarins. As well, the

increase of scopoletin-peroxidase could explain the decrease of scopoletin at the end of the PPD time course (Figure 4.21).

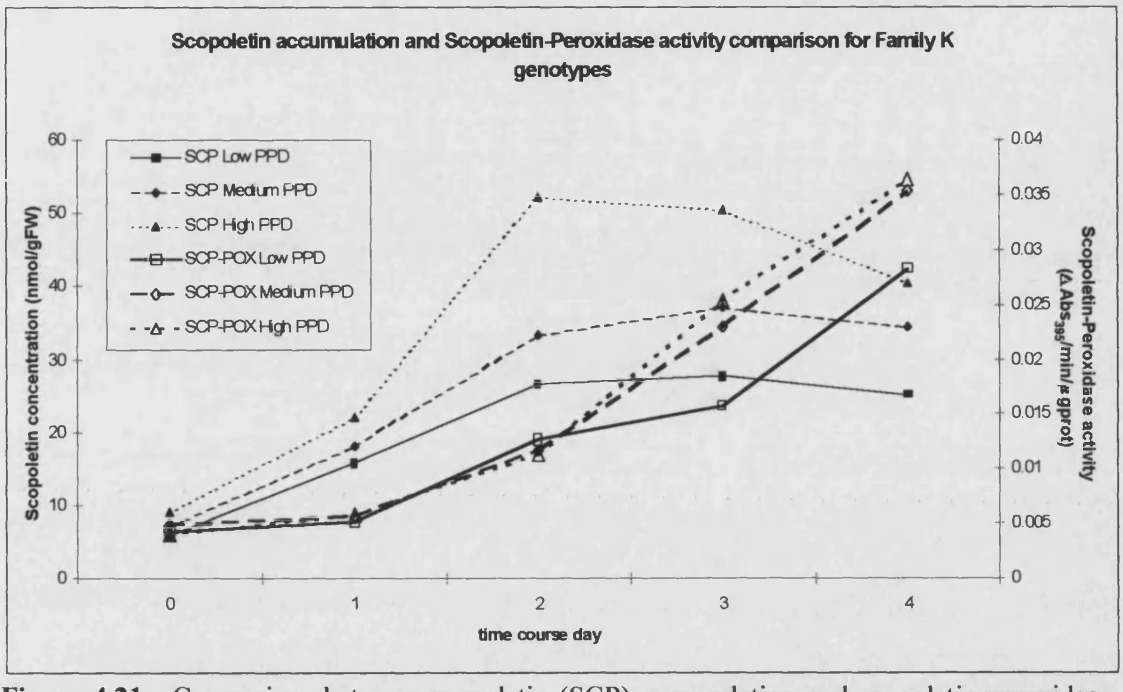


Figure 4.21. Comparison between scopoletin (SCP) accumulation and scopoletin-peroxidase (SCP-POX) activity during a time course of four days, between a selected percentage of Family K population with different responses to PPD.

4.3 DISCUSSION

The results presented here describe the activity of enzymes, PAL, POX, CAT, PPO, Glucanase, chitinase, characterized as induced by wounding (Bowles 1990; Cabello et al. 1994; Wenham 1995).

Results obtained with PAL did not confirm previous reports on the correlation between this enzyme and PPD response, where PAL was shown to have a peak in activity at 48 h of injury (Data et al. 1984; Rickard 1985; Tanaka et al. 1983; Tanaka et al. 1984; Uritani et al. 1983).

However, PAL activity was only measured on one June 1999 group of samples, when whole roots were used as samples. New experiments should be performed to evaluate and confirm PAL activity in slices or cassava root portions in a large number of cultivars showing contrasting PPD responses.

Tables 4.1 and 4.2 summarise the results obtained for the average of enzyme activity and for the frequency of peaks for the three PPD susceptibility levels, respectively. PPO, POX and SCP-POX were the most PPD- related enzymes. PPO, as has been previously reported (Kato et al. 1991), was positively correlated with tissue browning. As it was done in chapter three, tables 4.1 and 4.2 will be used to determine the most appropriate day for evaluating enzymatic activity. For PPO and SCP-POX day four revealed the general trend of all cultivars, while for POX days two or three reflect the general tendency of the group of samples which presented the more defined PPD reactions (December 1999 group where the PPD level was better characterised)

Activity for chitinases and β-1,3-glucanase showed differences between PPD levels, but these enzymes did not prove to be of interest in PDD, since the start of their activity increased was observed between days four and five.

Peroxidase isoforms expressed during PPD were determined. Most of the isoforms were anionic and some of them showed an increase of their activity and were only present in the susceptible cultivar. This observation could lead to the determination of a possible PPD marker but further characterizations are needed. These results concur with previous studies, even though the isozyme separation was made by means of molecular weight. Plumbey and Hughes (1982) reported the presence of six peroxidase isozymes in non deteriorated root and seven in deteriorated roots (24 h after wounding). Plumbley et al. (1981) and Marriot et al. (1980) also reported that changes in peroxidase isoforms are accompanied by increase in the peroxidase activity. Marriot suggested that peroxidase activity may be associated with formation of lignin-like material from phenols and may influence in the development of the pigment associated with vascular streaking.

Isoforms of scopoletin-peroxidase accumulated over the time course but they were present in the low and high PPD-response cultivars, and unlike the peroxidases detected with AA and DHBS no candidate bands for enzymatic markers were found.

The correlations among metabolites, enzymatic activities and between them lead to interesting results and conclusions. Positive correlations could be detected among biochemical measurement that show the best results as markers for the PPD-response, for instance the correlation between PPD-marker and SCP-POX.

ENZYMES ACITVITIES DURING THE DETERIORATION AMONG PPD LEVELS								
SAMPLE GROUP	TIME COURSE DAY	PAL	CAT	PPO	POX	SCP-POX	B-1,3-GLUCANASE	CHITINASE
JUNE 1999	0					x		
	1	M				x		
	2		H			x		M
	3			L		x		
	4		M -H			x		
	5		L			x		L
	6	L -H			L	x		
	7			M -H	M -H	x	L -M -H	H
DEC 1999	0	x					x	x
	1	x	M				x	x
	2	x	M -H		L -M -H		x	x
	3	x	M	L			x	x
	4	x	M	M -H		L -M -H	x	x
FAMILY K	0	x	x				x	x
	1	x	x				x	x
	2	x	x		M		x	x
	3	x	x		L -H		x	x
	4	x	x	L -M -H		L -M -H	x	x

Table 3.1 Summary of the results obtained for the average of enzyme activity for the three PPD susceptibility levels: high (**H**), medium (**M**) and low (**L**). The position of the convention used to designate the PPD level indicates the day of the storage time course at which the maximum concentration of secondary metabolite is reached. **x**:enzyme not evaluated

FREQUENCY OF PEAKS DURING DETERIORATION AMONG PPD LEVELS								
SAMPLE GROUP	TIME COURSE DAY	PAL	CAT	PPO	POX	SCP-POX	B-1,3-GLUCANASE	CHITINASE
JUNE 1999	0	L	H			x		M
	1	L-M		L		x		
	2					x		M
	3	L	H			x		L
	4	L	M			x		
	5	H	L			x	L	H
	6	L			L	x	L	
	7	L-M		M -H	M -H	x	L-M -H	
DEC 1999	0	x					x	x
	1	x			M		x	x
	2	x			L-H		x	x
	3	x	M -H			L	x	x
	4	x	L-H	L-M -H		L-M -H	x	x
FAMILY K	0	x	x				x	x
	1	x	x		M		x	x
	2	x	x				x	x
	3	x	x		L-H		x	x
	4	x	x	L-M -H		L-M -H	x	x

Table 3.2 Summary of the results obtained for the average of enzyme activity for the three PPD susceptibility levels: high (**H**), medium (**M**) and low (**L**). The position of the convention used to designate the PPD level indicates the day of the storage time course at which the highest frequency of peaks is reached. There are cases in that the highest value of peaks frequency is the same for different days of the storage time course. **x**:enzyme not evaluated

CHAPTER 5

ENHANCEMENT OF THE UTILITY OF THE CASSAVA MOLECULAR GENETIC MAP

5 ENHANCEMENT OF THE UTILITY OF THE CASSAVA MOLECULAR GENETIC MAP

5.1 INTRODUCTION

The genes controlling quantitative traits usually do not lend themselves to uncomplicated classical genetic analyses. This is on account of the confounding effects arising from the complex nature of their genetic inheritance. These complexities could be as a result of gene - by - gene and/or gene - by – environment interactions. In order to overcome this problem, more powerful methods of analyses like molecular marker-assisted assays are usually employed. Molecular genetic maps constitute a potent means for analysing and genome location of complex traits and also identifying quantitative trait loci (QTLs) (Tanksley et al. 1993).

Fregene *et al.* (1997) constructed a molecular genetic linkage map of cassava based on a F_1 population (90 plants) developed from a cross between two elite cassava lines. Both parents are heterozygous on account of the large number of the diverse cassava cultivars that make up their pedigrees. The female parent, MNGA 2 (TMS 30572), is an improved cultivar developed at the International Institute of Tropical Agriculture (IITA), Nigeria. It is characterized for its high tolerance to the African cassava mosaic disease (ACMV) and cassava bacterial blight (CBB). The resistance to ACMV might be the result of introgression of a part of the *M. glaziovii* genome into the breeding line. The male parent, CM 2177-2 (Ica-Cebucan) was developed at CIAT. It is characterized by high photosynthetic rates, good cooking quality and tolerance to the cassava mealy bug and CBB (Fregene et al. 1997). Additionally MNGA 2 presents medium tolerance to PPD and CM 2177-2 is highly susceptible to PPD. The segregation data of 158 RFLP, 30 RAPDs, 3 microsatellites and 4 isozymes single dose markers were used to construct female parent based map using the MAPMAKER computer package. These formed a total of 20 linkage groups covering 931.6 cM of the genome with the linkage groups named alphabetically. . Additionally, the segregation data of 107 RFLP, 50 RAPDs, 1 microsatellite and 1 isozyme single-dose markers were used to construct a male parent based framework map made up of 24 linkage groups and covering a total distance of 1220cM of the genome.

The F_1 population used to construct the genetic map (144 genotypes) was evaluated for PPD in replicated trials at two different environments in order to identify QTLs (Cortes et al., in press). The probes used in this study were isolated from a cDNA library constructed from roots of MNGA1 48h after harvesting (Beeching et al. 1997). These probes coming from an expression library therefore corresponded to genes involved in wound responses: phenyl alanine ammonia lyase (PAL1), catalase (CAT1a and CAT 1b), hydroxyprolin rich glycoprotein (HRGP1), 1-aminocycloprpane 1-carboxylase ACCOX1), ß-1,3-glucanase (GLU), RNA polymerase subunit (cRNA-polIPCR), aspartic protease (cASP-1), cysteine protease inhibitor (cCPI-2) and a partial cDNA for serine/threonine protein kinase (cPK). Additionally, two other probes, phenyl alanine ammonia lyase (MEPAL) and peroxidase (MPEX1), were kindly provided by L. P. Pereira, University of Guelph, Guelph, Canada. Seven of the wound responses related genes segregated in the progeny. On the female map, the HRGP gene fell within the linkage group E; ACCOX in P; GLU in H and MEPX in L. For the male map, RNA and MEPAL were located on the UA group and PAL in UH.

The identification of QTLs had been used as an approach to study PPD in two different ecosystems (Cortés et al., in press). These QTLs were identified on a more saturated version of the cassava molecular genetic linkage map made up of 240 RFLPs, 100 RAPDs, 5 isoenzymes and 85 SSRs using the single-point and interval analyses of the QGene program. Seventeen significant QTLs related to PPD were identified (Table 5.1).

Marker	Location	Linkage group	Parent source	R2	Probability	Additivity
GY138	Quilichao	NgU	Nga2	0.12	0	5.36
rGY164	Quilichao	NgU	Nga2	0.09	0.0004	4.97
rNI1.C2	Quilichao	CmC	Nga2	0.13	0.0008	4.94
rSSR83	Palmira	NgP	Nga2	0.09	0.0012	11.18
CDY131	Palmira	NgX	Nga2	0.13	0.0025	13.15
rK16d	Quilichao	NgG	CM 2177-2	0.11	0.0026	-5.47
GY120	Palmira	CmL	CM 2177-2	0.11	0.0028	-11.91
SSR6	Quilichao	NgU	Nga2	0.08	0.0032	4.11
SSR6	Palmira	NgU	Nga2	0.07	0.0045	9.29
GY202	Palmira	NgL	Nga2	0.05	0.0057	8.02
rM5a	Palmira	NgX	Nga2	0.10	0.0070	11.73
rGY22-1	Palmira	CmA	CM 2177-2	0.09	0.0078	-11.20
AC-1	Palmira	NgM	CM 2177-2	0.10	0.0082	-10.95
rGY26	Quilichao	CmC	Nga2	0.09	0.0082	3.89
rE14b	Palmira	NgJ	CM 2177-2	0.10	0.0091	-11.76
RHRGP	Palmira	NgE	CM 2177-2	0.05	0.0094	-7.83
CDY123a	Quilichao	CmE	CM 2177-2	0.09	0.0094	-5.10

Table 5.1 QTL markers associated with PDD (Cortés et al., in press)

5.2 RESULTS

The identification of QTLs requires the scoring of segregation data from the entire population of the cross used to generate the genetic map. In our case it was not possible to assay the complete offspring because the large number of samples was unwieldy. Due to this logistic factor we were able to sample only a representative percentage of the population. The PPD response of the family K (whose F_1 progeny was used to construct the cassava molecular genetic linkage map) had been scored in 1998 in two different ecosystems (CIAT Palmira and CIAT Quilichao). Comparing the distribution of the PPD response in the two different ecosystems, (Figs. 5.1 and 5.2), CIAT-Palmira was chosen as sampling site because the deterioration data followed a normal distribution curve. The genotypes from the extremes (15 individuals) and the middle (five individuals) of the normal distribution were selected for use in the current study. Also, from within the three different groups, the genotypes showing the minimal standard deviation between replications were chosen.

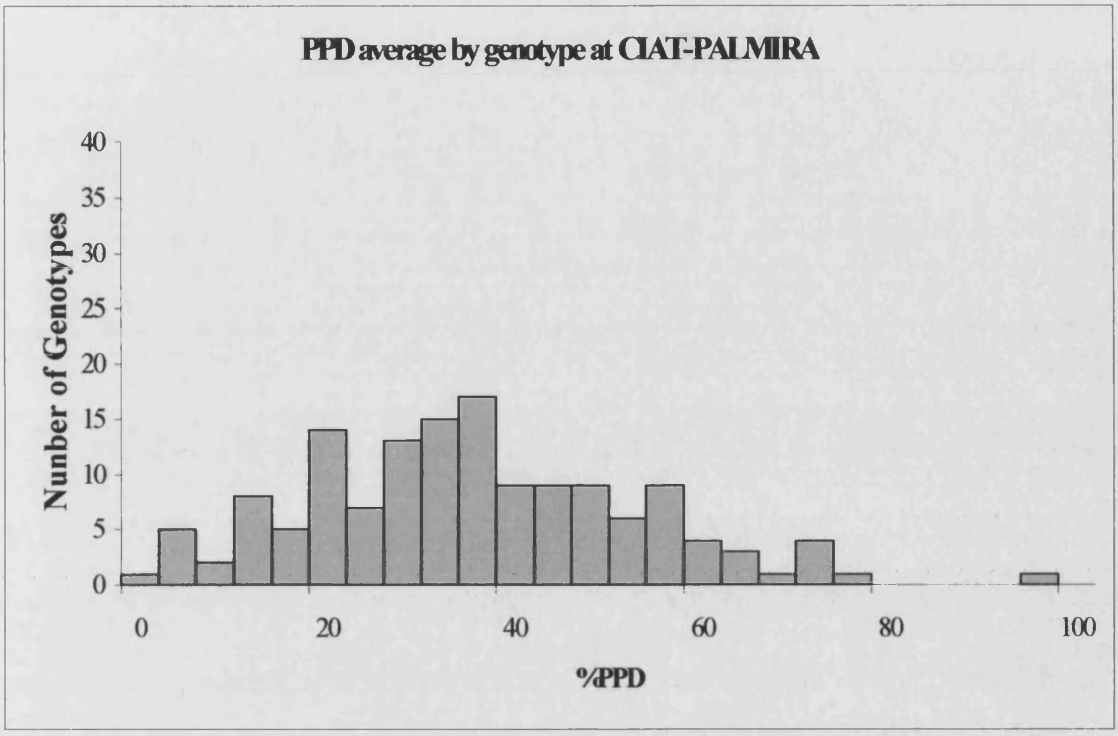


Figure 5.1 PPD response distribution of Family K at CIAT-Palmira in 1998

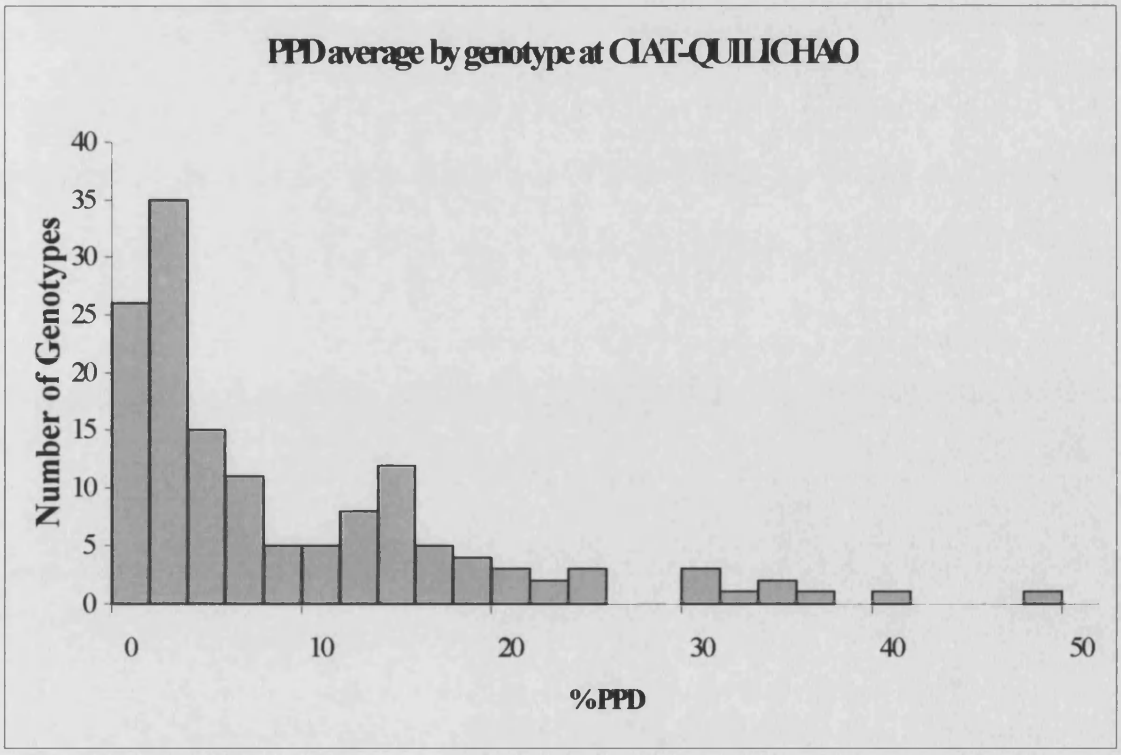


Figure 5.2 PPD response distribution of Family K at CIAT-Quilichao in 1998

The following table (5.2) summarises the genotypes selected and their PPD scores in the two sampling sites.

LOW PPD RESPONSE			MEDIUM PPD RESPONSE			HIGH PPD RESPONSE		
Genotype	%PPD Palmira	%PPD Quilichao	Genotype	%PPD Palmira	%PPD Quilichao	Genotype	%PPD Palmira	%PPD Quilichao
K-4	3.53	3.91	K-5	33.54	13.44	K-15	62.16	15.89
K-10	7.27	19.02	K-44	32.84	2.75	K-38	56.34	16.55
K-13	24.66	24.23	K-52	32.11	3.96	K-46	66.16	22.92
K-23	14.68	14.32	K-108	34.49	3.55	K-85	54.01	7.79
K-41	14.23	1.41	K-132	31.10	2.85	K-99	71.20	20.34
K-65	9.80	3.94				K-110	72.45	32.54
K-67	15.03	1.68				K-120	55.15	15.67
K-70	13.73	2.71				K-125	50.52	3.10
K-73	6.63	1.73				K-136	56.21	13.54
K-77	15.37	1.73				K-139	75.81	9.54
K-93	7.29	2.06				K-142	56.11	7.03
K-94	10.28	3.28				K-145	74.01	20.21
K-127	7.09	4.04				K-146	55.43	13.9
K-129	15.20	2.00				K-147	63.20	24.99
K-138	19.68	3.21				K-149	72.47	3.91

Table 5.2 Family K genotypes chosen for sampling and their PPD score in the two different ecosystems, Palmira and Quilichao.

The associations between molecular markers and the secondary metabolites and enzyme activity measurements were determined by the single point analysis method of QTL detection. The group of molecular markers included the markers used by Cortés et al. (in press) to determine the PPD QTLs. The single point analysis calculates a linear regression between the number of recessive alleles (x axis) and the value of the quantitative trait (y axis). In our case, the quantitative trait was the concentration and enzymatic activity of the secondary metabolites. The single point analysis also determines the significance of the intercept and the slope for each regression model. The intercept refers to the average value for the trait when there are no dominant alleles, and the slope refers to the allelic effect. The linear correlation coefficient (R^2), which is

also obtained from the analysis, quantifies the percentage of the variance of the trait being explained by the number of recessive alleles.

The values for the evaluated traits (concentration of scopoletin, scopolin, esculin, PPD-marker and soluble phenolic content, and activity of peroxidase, polyphenol oxidase and scopoletin-peroxidase) are not represented by just a single value. One possible way to unify the five values into one in order to facilitate the single point analysis is the calculation of the area under the curve described during the deterioration time course, but this value was not used. One statistical argument for not using the curve area value is its additive effect on the model variance as the mere calculation of the area under the curve formula generates a source of variance. It is not accurate to add this variance to the significantly high variance between repetitions observed in the ANOVA analysis (appendix 8.2.4). The second argument is of a biological nature. We measured the accumulation of metabolites and changes in enzymatic activity, which are continuous traits. The way we measured the trait during the time course was very discrete (one measurement every 24 hours). Thus, we were not able to accurately determine the real progress of the trait. After that, we decided to use the measurement of one day of PPD time course as the value for the linear regression analysis. It was decided that the most appropriate feature to choose the time course day was the day at the maximum concentration of the secondary metabolite or enzymatic activity. In order to do that, the average of the peak frequency among the 35 genotypes evaluated was calculated (Fig 5.3). Afterwards, day three was chosen for peroxidase and scopoletin, and day four for polyphenol oxidase, scopoletin-peroxidase, scopolin, esculin, PPD-marker and soluble phenolic content. In the case of peroxidase days one and three showed the same peak frequency, but day three was chosen because it is closer to the other traits' trends and better represents the general trend of the different PPD levels in the Family K samples group (see appendix 8.4.4).

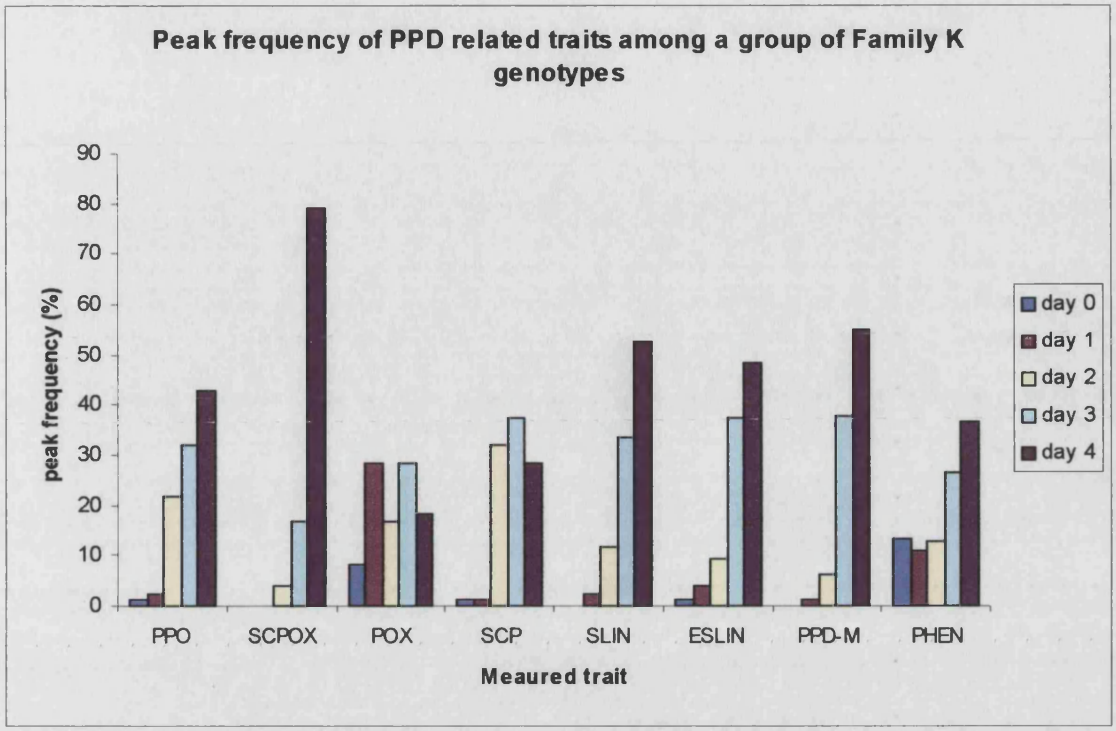


Figure 5.3 Averages of the peak frequencies of PPD-related traits among 35 family K genotypes. These traits, enzymatic activity of polyphenol oxidase (PPO), scopoletin-peroxidase (SCPOX) and peroxidase (POX), and concentration of scopoletin (SCP), scopolin (SLIN), esculin (ESLIN), PPD-marker (PPD-M) and soluble phenolic content (PHEN), were evaluated following a storage time course of four days. .

After running the single point analysis, the linear regression models showing a high significance for both intercept and slope were selected. Table 5.3 summarises the results of the single point analysis, showing the value of the linear correlation coefficient in the models where the intercept and the slope were highly significant. Once the relevant regression models were selected, the traits associated with the molecular markers were pointed out on the female linkage cassava map developed by Fregene *et al.*, 1997 (Fig. 5.4). This was done with the purpose of easily visualising on the different linkage groups of the map. The molecular markers highlighted with an asterisk (*) in table 5.3 were not included in the female cassava linkage map.

Looking at the female genetic map and the associated PPD related traits two linkage groups (F and I) show interesting zones. Marker GY80 in linkage group F was associated with scopoletin, peroxidase, PPD-marker and esculin. The first three PPD traits proved to play a determinant role in the PPD response, which make this zone an interesting point for further study. Additionally, other markers in the same linkage group showed association with peroxidase, scopoletin-peroxidase, scopoletin and PPD-

marker. It would help to further saturate this map with other molecular markers in the hopes that a fine mapping of these regions of the genome could be achieved. The same suggestion could be applied to the linkage group I, where marker AC1 was significantly associated with peroxidase, scopoletin-peroxidase and scopoletin, R1 with peroxidase and D5a with scopoletin. It might be expected that markers associated with the same trait were linked, for example GY164 and GY224 in group A and GY93 and GY143 in group O with scopolin, and GY80 and GY204 in group F with PPD-marker. But in our case it was not observed because the number of genotypes evaluated for the biochemical PPD related traits was very low. It is also plausible to conclude that such an association between these molecular markers would have been achieved with a denser map, also underscoring the need for the fine mapping of these regions of the cassava genome. It is also seen from Table 5.2 that another marker, GY27 (not included in the female map), just like AC1 is associated with peroxidase, scopoletin-peroxidase and scopoletin. Another interesting observation is the association of peroxidase and scopoletin with five markers (K2a, GY27, GY219, GY80 and AC1) showing relatively high linear correlation coefficients.

Additionally, the PPD response of the Family K in two different localities was evaluated for two consecutive years. The localities were CIAT-Palmira (ECZ 4, mid altitude tropics) and CIAT-Quilichao (ECZ 2-4, mid altitude tropics and acid soils). Figure 5.5 shows the difference of the frequencies of the PPD response. These results reconfirm the strong GxE interaction in the expression of post harvest deterioration. Even though the differences between the climatological conditions in the two following years were not significant, it is very surprising the change of the PPD response in Palmira from 1998 to 1999. ANOVA analysis (appendix 8.7) showed significant differences between localities and season except for Quilichao during 1998 and 1999.

	MM	PPO	SCPOX	POX	SCP	SLIN	ESLIN	PPD-M	PHEN
*	CBB4	0.16					0.17	0.17	
	GY122							0.12	
	GY128		0.18						
	GY141	0.29				0.18	0.26		
	GY119					0.37			
	GY197						0.22	0.26	
	GY203						0.4	0.27	
	GY204							0.22	
	GY42							0.27	
	GY28						0.25		
	GY205								0.19
	GY31						0.39		
	GY37		0.25						
	GY212					0.2			
	GA-127		0.19						
	GY217					0.17			
	GY219			0.13	0.12				
	GY153					0.22			
	GY223					0.35			
	rGY145					0.23			
	rGY164					0.17		0.14	
	rGY167				0.13				
	rGY172								0.14
	rGY180								0.15
	rGY75						0.2		
	rGY208				0.23				
	rGY74					0.17			
	rGY108						0.19		
	rGY81						0.19		0.14
	rGY220						0.14		
*	AM18							0.21	
	rD5a				0.13				
*	H14b								0.23
*	K2a			0.30	0.28				
	R1			0.23	0.21				
*	rF19b								0.23
*	ri14b								0.29
*	rP1a								0.25
*	GAGG5					0.23			
*	S2							0.2	0.21
	AC-1		0.23	0.21	0.28				
*	CPY79	0.2							
*	GY5								0.3
	GY93					0.26			
	GY101-1								0.17
	GY71					0.27			
	GY80			0.25	0.35		0.22	0.26	

Table 5.3 Summary of the significant linear regression coefficients found by the single point analysis between the molecular markers used for the construction of the linkage cassava map and PPD related traits. Values in the table correspond to the linear regression coefficients in each regression model between molecular markers (MM), polyphenol oxidase (PPO), scopoletin-peroxidase (SCPOX), peroxidase (POX), scopoletin (SCP), scopolin (SLIN), esculin (ESLIN), PPD-marker (PPD-M) and soluble phenolic content (PHEN). (*) molecular markers not included in the female cassava linkage map. Table continues next page.

	MM	PPO	SCPOX	POX	SCP	SLIN	ESLIN	PPD-M	PHEN
	GY44						0.24		
	GY83					0.28			
*	GY105					0.26			
*	GY77	0.21							
*	GY42						0.21		
	GY53								0.22
*	CDY123b								0.32
*	GY52	0.16					0.17	0.17	
	CDY128							0.12	
*	GY120		0.18						
*	rGY87					0.22			
*	rGY22-1		0.22						
*	rGY27		0.19	0.26	0.23				0.18
*	rGY99								0.28
*	rCDY44	0.24							
*	rBEST-2					0.6			
*	G9				0.30				
*	i18b		0.23						
*	L20				0.13				
*	O20			0.17			0.2		0.14
*	U1		0.18						
*	rI2							0.12	
*	rI4A					0.14			
*	rK9A					0.18			
*	rQ11							0.19	
	nGY143					0.26			
*	nrGY66					0.2			
*	nrGY67		0.26						
	PASK1		0.3						
*	11					0.26	0.12		
*	382		0.27						
*	277		0.18					0.2	
*	r22'							0.24	
*	r11								0.21
	rS137		0.24						
*	r184					0.15			0.13
*	r277	0.21							
*	r449						0.31		0.2
*	377							0.2	0.18
*	r198					0.25			
*	MEPX							0.13	
*	PAL			0.15					
*	MEPAL		0.12						
*	rMEPX							0.13	
*	rPAL			0.15					
*	rMEPAL		0.12						

Table 5.3 Summary of the significant linear regression coefficients found by the single point analysis between the molecular markers used for the construction of the linkage cassava map and PPD related traits. Values in the table correspond to the linear regression coefficients in each regression model between molecular markers (MM), polyphenol oxidase (PPO), scopoletin-peroxidase (SCPOX), peroxidase (POX), scopoletin (SCP), scopolin (SLIN), esculin (ESLIN), PPD-marker (PPD-M) and soluble phenolic content (PHEN). (*) molecular markers not included in the female cassava linkage map.

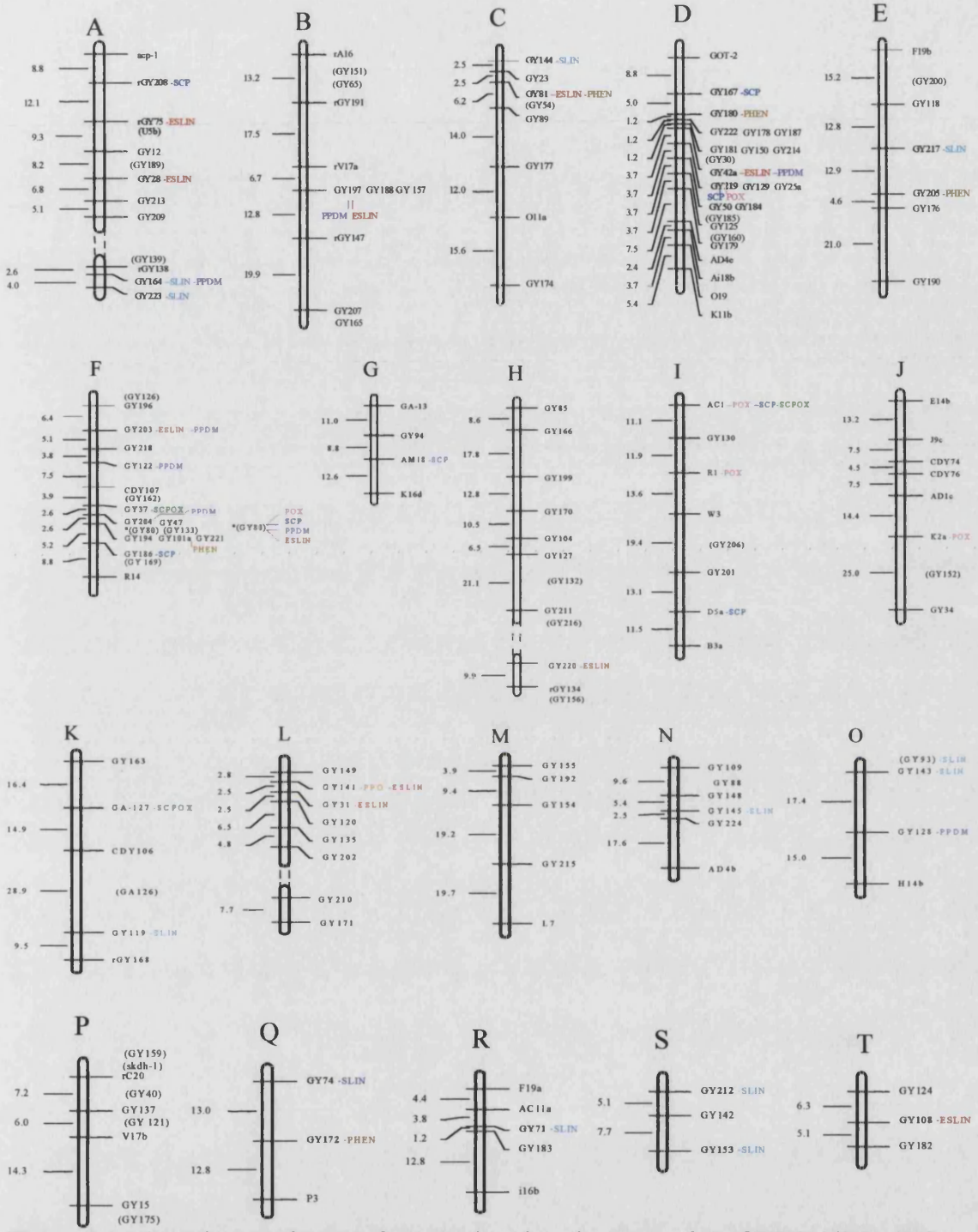


Figure 5.4 Molecular genetic map of cassava based on the segregation of RFLP (CDY cDNA, GY genomic), microsatellite (GA), isozyme (acp, skdh and got) and RAPD markers (prefix A-AP through Z) in gametes of the female parent (MNGA 2), in a F_1 cross with CM 2177-2 (male parent) by 90 individuals (Fregene *et al.* 1997). Markers with suffix a represent duplicated loci, markers adjacent to horizontal lines belong to the framework (LOD>2.0) map, markers following on the same line co-segregate, while the remaining markers (in parenthesis) are placed in the most probable interval. Map distances (shown in the left) are indicated in Kosambi map units. PPD-related traits significantly associated with the markers by the single point analysis are indicated next to the corresponding marker. PPD related traits are polyphenol oxidase (PPO), scopoletin-peroxidase (SCPOX), peroxidase (POX), scopoletin (SCP), scopolin (SLIN), esculin (ESLIN), PPD-marker (PPD-M) and soluble phenolic content (PHEN).

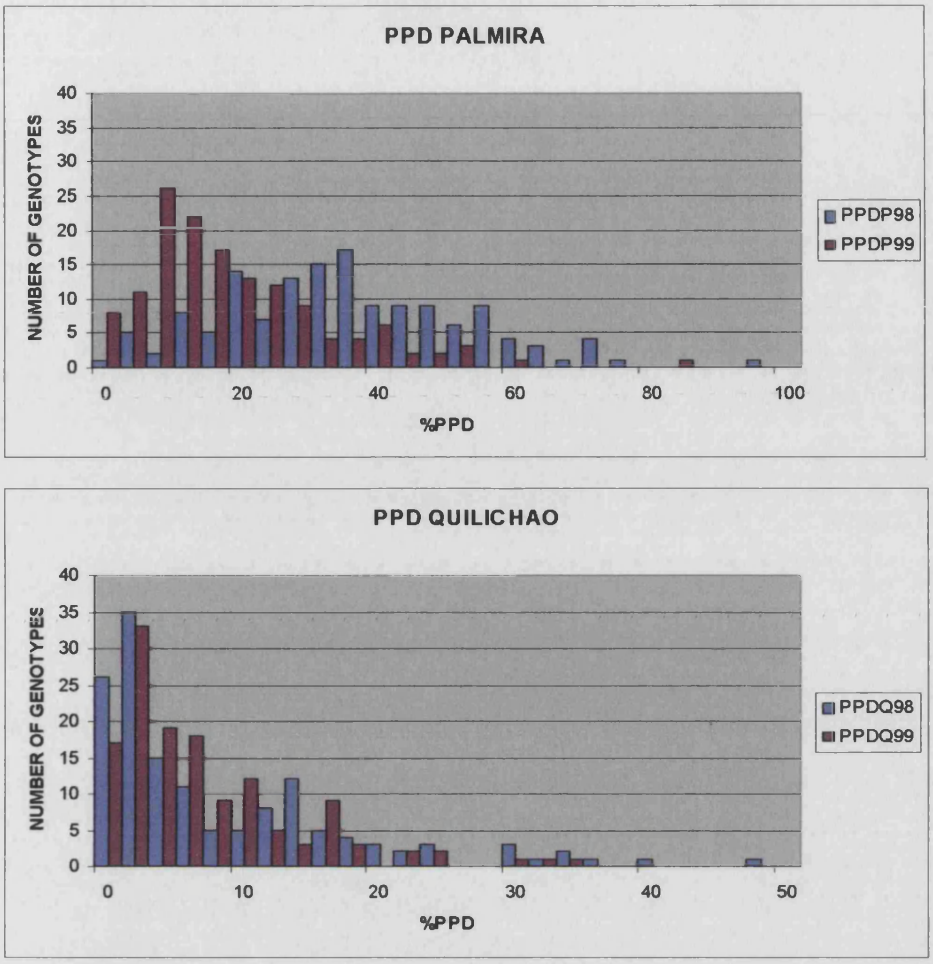


Figure 5.5. Comparison of distributions of the PPD response amongst the Family K mapping population in two contrasting agro-ecologies in Colombia in 1998 and 1999.

5.3 DISCUSSION

The most remarkable observation, based on the ANOVA analysis presented in the previous section, was the confirmation of the significant genotype by environment interaction (GxE) influencing PPD.

The evaluation of the PDD response of the mapping progeny showed confusing results, there was a surprisingly high frequency of very low PPD scores, bearing in mind that the parentals had medium and high responses to PPD. These low scores may be the result of the strong GxE affecting the expression of PPD.

Based on the PPD phenotypic evaluation of family K in two sites, CIAT-Palmira and CIAT Quilichao, in 1998, Cortes et al. (in press) calculated a heritability value of 60 %

indicating that part of the phenotypic variation observed in the PPD response had a genetic component. Comparable values for heritability, intermediate to high, were reported by Iglesias et al. (1996) in a study conducted in three different agroecologies between different genotypes. Contrasting results were found by Okogbenin and Fregene (in press). They evaluated the PPD response of the family K and other root quality traits in two different agroecologies, CIAT-Palmira and CIAT-Quilichao, during 1998 and 1999. The results showed significant differences between genotypes for all traits except for PPD. As well, they estimated a broad range of heritability for the traits, being PPD the lowest value (7%). Based on this low heritability value, they suggested a high non-genetic influence on the expression of PPD in the mapping population, which concurs with their finding of non-significant variation for PPD between the populations. Additionally, they detected 11 QTLs for PPD, eight in Palmira and three in Quilichao. The finding of QTLs associated with one locality may be due to the very significant GxE interaction. The 11 QTLs explained a phenotypic variance between 8 and 15 %. The strongest effect on PPD was detected for the QTL *phdD.1* on linkage group D for reduced PPD. The single point analysis, between the molecular markers and the biochemical quantifications (secondary metabolites and enzyme activity), presented in section 5.2, found association of some of the molecular markers in the linkage group D with scopoletin, esculin, PPD-marker, soluble phenolic content and peroxidase. Another QTL determined by Okogbenin and Fregene causing increase in PPD was found in linkage group E, where we found molecular markers associated with scopolin and phenolic content. Another interesting finding in the Okogbenin and Fregene study was the highly significant positive correlation between PPD and dry matter content which reconfirms the observations in the four progenies mentioned in chapter three.

The QTLs determined by Cortes et al. (in press) were associated with the effect between environments reinforcing the observation that the environmental variation was high.

Linkage group D in the molecular map of cassava seems to be a group of special interest, which has many markers and low recombination. Furthermore, Jorge et al. (2001) found a QTL for resistance to CBB in this linkage group. It is interesting that this QTL is linked with the molecular marker GY 219, which in our study resulted highly significantly correlated with peroxidase and scopoletin, both related with the response to pathogen attack, production of protective barriers and antimicrobial activity respectively. Another QTL for CBB resistance determined in this study was associated with the linkage group F correlated with the molecular markers GY 37 and GY 186,

which by the single point analysis were determined to relate significantly with scopoletin peroxidase and scopoletin respectively. Jorge et al. also found a QTL in the linkage group I associated with the molecular marker AC1, which proved to be correlated to scopoletin, peroxidase and scopoletin-peroxidase. Cortes et al. determined, as well, a QTL for PPD linked with the molecular marker AC1.

As the PPD response involves different biochemical process, which involve numerous genes, traditional breeding strategies will need to use methods for manipulating quantitatively inherited traits. The utility of the information coming from QTLs determination depends on the quality of the QTL study and the proportion of determinant QTLs successfully detected. Cassava breeders must pay especial attention to the previous observations, because the finding of non-significant variation for PPD between the F1 mapping population (Okogbenin and Fregene, in press), suggests that the cross may not be appropriate for PPD QTL determination. Another consideration that cassava breeders must have in mind is that large populations are required to gain sufficient statistical resolution to map multigenic and/or quantitative traits (Mazur and Tingey, 1995), a population with only 144 individuals may not be large enough to gain that statistical resolution. At last, in the cases when the associations between molecular markers (RFLP classes) and phenotypic evaluations are low, there is a need for precise methods of measuring phenotypic differences in quantitative characters, which constitutes a prerequisite for successful QTL mapping (Bonierbale et al 1993)

Developing comparative maps with related species or species where the probes for wound-related genes have been characterised, might be a potential tool for the elucidation of the genetics and function of the genes expressed during PPD.

CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

The visual score method traditionally used to evaluate PPD has been considered to be subjective and non-reliable, for that reason one of the aims of this study was to establish a biochemical method which provided more objective and accurate measurements related to PPD. In addition, this is the first study of the accumulation of secondary metabolites and enzymatic activity, related to PPD performed on a large collection of cassava cultivars.

Results showed that biochemical markers were identified for PPD, some previously reported and some new. Nevertheless, results showed that biochemical quantifications revealed a high variability between the tuberous roots of even the same cultivar and between different harvesting seasons and this contributes to the high standard deviations for all measurements.

Consequently, it was assumed that both methods, visual and biochemical, determine features of the post-harvest physiological deterioration process, and are subjected to the same inter-root and inter-season variation which is influenced by environmental effects, as are many other traits in cassava, like dry matter content. This major effect of the environment and the variability in PPD scores has been observed in CIAT for many years (T. Sánchez, pers. comm.). For this reason, the importance of having a controlled environment, like a controlled-environment chamber for the PPD evaluation of cultivars and the measurement of biochemical markers is once again highlighted.

Differences in the results between the Bath group samples and the CIAT group samples, including the family K may be caused by the storage time to which the Bath group of samples was submitted before the measurements. This is the reason why some of the biochemical measurements in the Bath group of samples are higher than in the other group samples or why hydroxycoumarins like esculetin could only be detected in this group sample. Also, in the June 1999 group samples another factor contributed to the results obtained, whole roots were used, causing the dilution of the biochemical markers. Regarding the family K group of samples, even though the statistical analysis showed differentiation among PPD level groups or among cultivars, both parents of the cross showed a medium (female parent) to low (male parent) susceptibility to PPD, and for this reason no useful segregation of the PPD response was expected among the progeny. A cross between parents showing a more contrasting response to PPD could

be more appropriate. Regarding all results, the December 1999 sample groups showed the clearest results.

Despite the inter-root and inter-season variability mentioned during the study, the visual score confirmed differences in the occurrence time and progress of the vascular streaking between cultivars. The histological observations confirmed the formation of tyloses and occlusions in xylem vessels cell walls described by Rickard et al. (1979) and Rickard and Gahan (1983).

The correlation between some biochemical measurements and the PPD susceptibility showed in this study could be a useful and objective tool for plant breeders for the evaluation of cassava cultivars for PPD response. The biochemical measurements (secondary metabolites or enzymes) showing the best and the worst correlations with the PPD response, and the data analysis will be discussed in more detail.

Regarding the data analysis, various statistical test and different variables were assayed. Analysing the results presented here, it is recommended to use direct measurements of secondary metabolites presence or enzymatic activity. The use of the area under the curve could result in misleading interpretations of the results since difference in results could be due simply to variations in the initial values (i.e enzymatic activities) among cultivars and not in PPD responses. The determination of peak frequencies was very useful in order to determine the day of higher expression or accumulation for enzymes and metabolites respectively. In this way, we can select an objective way the best day to realize the measurements for the PPD-markers.

Finally, it is recommended to work only with two clearly contrasting PPD level groups, low and high since the characterization of the PPD response is so subject to environmental fluctuations, the medium category cultivars sometimes behave as low and sometimes as high making more difficult the interpretation of the results.

The general trend in phenolic accumulation was not clear in any of the groups of samples studied, even though this test was to be a simple and very efficient way to measure the activation of the phenylpropanoid pathway in response to PPD. One reason for the difficult interpretation of the results may be due to the fact that the folin-ciocalteu reagent is not only specific for phenol since it can react with other components, such as aminoacids (Lowry et al. 1951). Nevertheless, this is a common characteristic of all methods for phenolic quantitative determination. To further understand the accumulation of phenolic compounds during the PPD response it is necessary to determine the localisation of the compounds in the different root tissues

and within the cell. The localisation of metabolites within the cell could be performed by means of confocal scan microscopy.

The fluorescent compounds observed in the root tissues under UV were identified as hydroxycoumarins (scopoletin, scopolin and esculin) as previously described by (Tanaka et al. 1983; Uritani et al. 1984a; Uritani et al. 1984b). The fourth hydroxycoumarin, esculetin, had not been described before (Buschmann et al. 2000). The fluorescence microscopy observations confirmed the increase of hydroxycoumarins after the first day of storage initially as the result of their accumulation in the cell wall xylem vessels, while in the later stages of the time course the coumarins spread to the storage parenchyma. Fluorescence microscopy showed accumulation of blue fluorescent compounds in the xylem vessels cell walls which then spread all over the vascular tissue.

Among the hydroxycoumarins, and based on the results obtained here, scopolin and scopoletin can be considered the most PPD-related secondary metabolites because of their higher accumulation. In addition, as previously mentioned in chapter 3, days 2-3 are recommended as the best days for the scopolin and scopoletin measurement. Based on the rapid progress of scopoletin production and the consequent deterioration, Wheatley (1982) suggested that the process is self-accelerating or autocatalytic.

The biosynthetic pathway of scopoletin in cassava remains unclear. Studies carried out by Wheatley (1982) in trying to determine the pathway were not conclusive since inoculation of probable intermediates (free phenolic acids) in pathways from cinnamic acid to scopoletin did not result in the increase of vascular streaking. This might suggests that the pathway from cinnamic acid to scopoletin does not correspond to these free phenolic acids. Probably scopoletin synthesis may occur via esters of these acids. Wheatley also inoculated cassava roots with extracts from acid-hydrolysed fresh cassava tissues. The assay did not show increased the vascular streaking in the fresh tested tissues. This suggests that no large reserve of scopolin is held in fresh tissues, which could be converted to scopoletin after harvest. This would be the most straightforward pathway conducting to scopoletin accumulation, as only the removal of the glucose molecule from scopolin by β-glucosidase is required.

The peaks of accumulation of scopolin after scopoletin may suggest, that scopoletin is not derived from scopolin during the occurrence of PPD. The opposite event, scopoletin as precursor for scopolin, might be more related with cassava. Elicitation of tobacco cells suspension cultures produced an early accumulation of phenylpropanoid

glucosyltransferase, along with the rapid synthesis and secretion of scopolin (Chong et al. 1999). In this assay scopolin was shown to represent a transportable form of scopoletin. It constitutes a way to protect scopoletin from highly reactive phenolic hydroxyl against cellular oxidases by means of O-glucosylation. The formation of soluble glycosides is generally assumed as a precondition for transport of phenolics to the apoplasm. This study also proved that the elicitation induced secretion of scopolin, intracellularly produced, allowing its cleavage by extracellular β-glucosidases and subsequent release of the reactive form scopoletin. This finding supported the thought that glycosilation may provide a pool of inactive and transportable forms of compounds that can be transformed to active form by β-glucosidases, as has been observed in roots of maize (Brzobohaty et al. 1993 as cited by Chong et al. 1999).

Another possible pathway for scopoletin biosynthesis was examined by Gutierrez et al. (1995) in sunflower. Enzyme assays demonstrated the presence of an elicitor-inducible methyltransferase, which methylated esculetin to give scopoletin. However, the caffeic acid o-methyltransferase activity, responsible for ferrulic acid synthesis, was also elicitated and the presence of the esculetin methyltransferase could not be considered effective evidence for the synthesis of scopoletin from esculetin. This possible pathway may not be applicable to cassava, since the detection of esculetin was not significative at the beginning of the storage time course.

TLC assays with the radical DPPH showed a wide range of bands with antioxidant activity. In tobacco cell cultures after elicitation, extracellular scopoletin, as well as caffeic and were rapidly metabolised by peroxidases implicating H_2O_2 consumption. Consequently, this compound may represent a potent antioxidant that can act as ascorbate or glutathione (Noctor and Foyer 1998).

Our results showed that free phenyl propanoids present in cassava, along with peroxidases could have a similar function acting as direct scavengers of H_2O_2 produced after wounding stress. As H_2O_2 has been considered a diffusible signal for plant defence reactions, free extracellular phenylpropanoids may be involved in the control of spread of H_2O_2.

In general, TLC proved to be a useful method for the secondary metabolite profile visualisation and for the tentative confirmation of their chemical nature, since specific stains for diverse families of compounds have been developed. However, TLC cannot be used as the only way for metabolite detection, as not all identified metabolites were

detected by this method. Considering the relative simplicity of TLC assays, it would be possible to use that method for the evaluation of large number of samples.

Rickard (1985) and Uritani (1998a) observed that the flavan-3-ols (+)-catechin and (+)-gallocatechin accumulated after four days of storage. These did not have high relation with PPD since vascular streaking starts two days after harvesting. In our study these flavan 3-ols accumulated slightly after two days of wounding stress induction. The accumulation of these catechins was more evident only after five days of storage. (+)-Catechin gallate accumulated in very low amounts compared to the other flavan-3-ols. These observations confirmed the low association between flavan-3-ols and PPD (Buschmann et al. 2000a). The decrease of flavan-3-ols after three days observed in some cultivars may imply the turnover of these compounds by oxidative processes, such as oxidation mediated by polyphenol oxidase (PPO), which was shown to occur in deteriorating cassava roots (Kato et al. 1991). The late and not very significant (in all cultivars) accumulation of flavan-3-ols did not make these compounds good candidates for PPD markers.

As it was observed with the accumulation of soluble phenolics, the tannin accumulation did not show a marked trend. This result might be expected looking to the obtained flavan-3-ols accumulation and knowing that Rickard (1985) proposed that flavan-3-ols can be metabolised into condensed tannins.

The so called "PPD-marker" is also highly recommended as a tool to measure the PPD response. However, its structure could not be determined. For future attempts to characterize it, it is recommend extracting it only from the root tissues showing clear symptoms of vascular streaking.

Although microbial deterioration in cassava starts after 5-7 days, anti-microbial compounds were detected in fresh slices and increase during storage time (Sakai et al. 1983, Uritani et al. 1984b and Rodriguez et al. 2000). These anti-microbial metabolites might play a role either as constitutive, preformed inhibitors or phytoncides (Mercier 1997), or as inducible compounds, phytoalexins, during physiological deterioration. The presence of anti-microbial substances in cassava roots would suggest the occurrence of a defence mechanism related to PPD that counters potential infection during harvesting, handling and storage of roots.

PAL was expected to be a good PPD marker. In sweet potato, in response to wounding (slicing) PAL activity markedly increase reaching a maximum 24 h after wounding

under light storage conditions (Singh et al. 1998). In lettuce PAL induction and synthesis and accumulation of phenolics occur before visual lesions of post harvest damage (russet spotting which mainly consist of brown spots) (Tomás-Barberán et al. 1997). PAL activity and the total soluble phenolic content were confirmed to change in response to post harvest physiological disorders (Ke and Saltveit 1988). However, results presented here were very variable and did not confirm observations of previous studies (Tanaka et al. 1983; Uritani 1998a; Uritani et al. 1984a). The importance of an environment-controlled chamber to study PPD-related characteristics was already pointed out and if its necessity was clear for the determination of secondary metabolites, it is even clearer for the determination of enzymatic activities. Besides PAL, catalase results were also very unclear and did not permit the description of a trend in its activity during the time course and correlation with the PPD-response.

In all studied groups of samples, PPO clearly showed a trend of parallel increase with the progress of post-harvest deterioration as have been reported by Data et al. (1984) and Kato et al. (1991), but it showed marked differences in activity values between different seasons for the low PPD susceptible cultivars. From the June 1999 group PPO showed to be one of the most PPD related enzymes as results of samples clearly showed and is one the most recommended for further studies on PPD. In addition, it is highly recommended, not only for the results obtained but also for the simplicity of the protocol used to measure it, as compared to the protocols used for catalase or PAL. Results obtained by Ke and Saltveit (1988) showed that the browning reaction in harvested lettuces may be largely due to the accumulation and oxidation of flavonoids, like (+)-catechin and (-)-epicatechin, and chlorogenic acid derivates, since the activity of PPO was 100 fold higher than the activity of PAL (Ke and Saltveit 1988).

Comparing PPO and POX as "PPD-markers", results showed, as was previously reported by Campos and de Carvalho (1990), that the activity for POX in response to PPD was higher that the activity of PPO. Ionically bound POX has been observed to highly correlate with the increase of russet spotting in lettuce, which is a physiological post harvest disorder (Ke and Saltveit 1988). Soluble POX may not be involved in the process of cell wall lignification, as cytochemical studies have shown that soluble POX exists in the inner fase of the tonoplast (Thomas and Jen as cited by Ke and Saltveit 1988), while the oxidation of phenolic precursors occurs in the cell wall.

Since scopoletin proved to be one of the most PPD-related metabolites and the fact that scopoletin has a high affinity for peroxidase (Schafer et al. 1971), which showed to be

correlated with PPD, SCP-POX may be an interesting marker and probably explains the decrease of scopoletin being caused by peroxidase. Scopoletin has a high affinity for peroxidase (Schafer et al. 1971), so it is probable that the decrease of scopoletin is due to oxidation by peroxidase. The peroxidative metabolism of scopoletin may have a direct defensive function as it has been suggested in tobacco (Goy et al. 1993 as cited by Edwards et al. 1997). Otherwise the function of the peroxidase may be to protect plants from the potential toxic effects of scopoletin. Furthermore, the capacity of scopoletin of being substrate for peroxidase has been used to develop methodologies to quantify hydrogen peroxide (Corbett 1989). Regarding all these results, it is important to bear in mind that the "scopoletin peroxidase" terminology implies only a certain specificity for scopoletin as a substrate and is not meant to imply that this is the only substrate altered by this enzyme or the only enzyme capable of altering scopoletin (Reigh et al. 1973).

No less important as PPD response markers are some enzymes related with respiration, catalases and peroxidases, which protect the plant from reactive oxygen species (ROS), such as superoxide (O_2-) and hydrogen peroxide (H_2O_2). These ROS are not only interesting because they could damage the plant cells, they are also involved in defence responses to wounding or pathogen attack. Glucanases and chitinases could be important for a microbial deterioration. It is necessary to develop a storage system that prevents contamination with fungi and bacteria. After five days of storage, microbial contamination starts. Notwithstanding the contaminated tissue was not used for the extractions, it is very important to avoid any source of contamination to make sure that the results are due only to physiological deterioration.

Molecular genetic maps constitute a potent means for analysing the genome location of complex traits and also identifying quantitative trait loci (QTLs) (Tanksley et al. 1993). Looking at the cassava female genetic map and the associated PPD related traits two linkage groups (F and I) show interesting zones. In these zones several biochemical markers were co-localized. The identification of these "hot-spots" of PPD-related markers in the map are very important since they can be incorporated in a marker-assisted breeding scheme.

The positive correlation between dry matter and PPD may be explained by the fact that humidity is required for metabolite transport, so those metabolites with antioxidant properties (water soluble like (+)-catechin and other antioxidants present in cassava roots like ascorbic acid) can not be diffuse throughout root tissue and consequently inhibit oxidative reactions.

PPD is a complex process, which involves several secondary metabolites and enzymes of different metabolic pathways. In this study, correlations between secondary metabolites or enzymes and the PPD process were clarified. Some correlations are still uncertain and the role of some enzymes or metabolites in the process is far from being elucidated. As Wheatley and Schwabe (1985), highlighted, "the resolution of all the outstanding uncertainties could be important in helping to understand and control one of the major problems associated with the utilization of cassava roots in the tropics."

CHAPTER 7

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7 BIBLIOGRAPHY

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CHAPTER 8

APPENDICES

8 APPENDICES

8.1 THIN LAYER CHROMATOGRAPHY (TLC)

8.1.1 TLC for detection of coumarins and flavonoids

CM 2177-2											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.03	NEU	orange				x	x	x	x	x	x
0.06	NEU	orange							x	x	x
0.08	UV(366nm)-NEU	blue-green				x	x	x	x	x	x
0.13	UV-NEU	blue	Scopolin			x	x	x	x	x	x
0.19	UV(366nm)-NEU	gray-green							x	x	x
0.29	UV(366nm)-NEU	blue			x	x		x	x	x	x
0.37	UV(366nm)	blue							x	x	x
0.44	UV(254nm)	gray	Catechin						x	x	x
0.48	UV(366nm)-NEU	blue							x	x	x
0.66	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.76	UV(254nm)	gray							x	x	x
0.79	UV(254nm)	gray		x	x	x	x	x	x	x	x

MCOL 22											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.05	NEU	green		x	x	x	x	x	x	x	x
0.08	NEU	blue					x	x	x	x	x
0.12	NEU	blue		x	x	x	x				
0.16	UV-NEU	blue	Scopolin		x	x	x	x	x	x	x
0.27	NEU	blue				x		x	x	x	x
0.36	NEU	blue		x	x	x	x	x	x	x	x
0.47	UV(254nm)	gray		x	x	x	x	x	x	x	x
0.66	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.74	UV(254nm)	gray							x	x	x
0.81	UV(254nm)	gray						x	x	x	x

MNGA 2											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.04	NEU	green		x	x	x	x	x	x	x	x
0.06	NEU	orange						x	x	x	x
0.07	UV(366nm)-NEU	blue						x	x	x	x
0.13	UV(366nm)-NEU	blue						x	x	x	x
0.17	UV-NEU	blue	Scopolin		x	x	x	x	x	x	x
0.67	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.76	UV(254nm)	gray							x	x	x
0.82	UV(254nm)	gray		x	x	x	x	x	x	x	x

MVEN 77											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.04	NEU	green		x	x	x	x	x	x	x	x
0.06	NEU	orange				x	x	x	x	x	x
0.12	UV-NEU	blue					x	x	x	x	x
0.14	UV-NEU	blue	Scopolin		x	x	x	x	x	x	x
0.28	UV(366nm)-NEU	blue-green			x	x	x	x	x	x	x
0.33	UV(254nm)	gray		x	x	x	x	x	x	x	x
0.39	UV(254nm)	gray	Catechin					x	x	x	x
0.43	UV(254nm)	gray		x	x	x	x	x	x		
0.59	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.71	UV(254nm)	gray						x	x	x	x
0.75	UV(254nm)	gray		x	x	x	x				

MBRA 12											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.04	NEU	green		x	x	x	x	x	x	x	x
0.11	NEU	green			x	x	x	x		x	
0.14	UV-NEU	blue	Scopolin		x	x	x	x	x	x	x
0.31	NEU	green		x	x	x	x	x	x	x	x
0.38	UV(254nm)	gray						x	x	x	x
0.42	UV(254nm)	gray	Catechin					x	x	x	x
0.62	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.78	UV(254nm)	gray		x	x	x	x	x			

MPER 183											
Rf	VISUAL	COLOUR	METABOLITE	DAY							
				0	1	2	3	4	5	6	7
0	NEU	orange		x	x	x	x	x	x	x	x
0.03	NEU	green		x	x	x	x	x	x	x	x
0.08	NEU	orange						x		x	x
0.14	UV(366nm)-NEU	blue		x	x	x	x	x			
0.17	UV-NEU	blue	Scopolin		x	x	x	x	x	x	x
0.27	UV(366nm)-NEU	blue		x	x	x	x	x	x	x	x
0.38	UV(254nm)	gray		x	x	x	x	x	x	x	x
0.44	UV(254nm)	gray		x	x	x	x	x	x	x	
0.45	UV(254nm)	gray	Catechin					x	x	x	x
0.64	UV-NEU	blue	Scopoletin	x	x	x	x	x	x	x	x
0.75	UV(254nm)	gray								x	x
0.80	UV(254nm)	gray		x	x	x	x	x	x	x	x

8.1.2 TLC for detection of non enzymatic antioxidants

CM 2177-2								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.37	x							
0.40		x	x		x	x	x	x
0.45	x	x	x					
s0.58	x	x	x	x	x	x	x	x
0.70					x	x		
0.77	x	x	x	x	x	x	x	x

MCOL 22								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.44	x	x	x	x	x	x	x	
0.47	x		x	x		x	x	x
s0.62	x	x	x	x	x	x	x	x
0.68	x	x	x	x	x	x		
0.75							x	x
0.82	x	x	x	x	x	x	x	x

MNGA 2								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.26		x	x					
0.36	x					x	x	
0.45	x							
s0.58	x	x	x	x	x	x	x	x
0.71						x	x	x
0.78	x	x	x	x	x	x	x	x

MVEN 77								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.33	x	x	x	x	x	x	x	x
0.38	x							
0.41	x	x	x	x	x	x	x	x
0.47	x	x		x	x	x	x	x
s0.59	x	x	x	x	x	x	x	x
0.68	x	x	x	x	x	x	x	x
0.73					x	x	x	x
0.79	x	x	x	x	x	x	x	x

MBRA 12								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.34	x	x	x	x	x	x	x	x
0.43	x	x	x	x	x	x	x	x
0.50	x	x				x	x	x
s0.62	x	x	x	x	x	x	x	x
0.71	x	x	x	x		x	x	x
0.79	x	x	x	x	x	x	x	x

MPPER 183								
Rf	DAY							
	0	1	2	3	4	5	6	7
0.43	x	x	x	x	x	x	x	x
0.46	x							
0.53	x	x	x	x	x	x	x	x
0.56	x						x	x
s0.64	x	x	x	x	x	x	x	x
0.70	x	x	x	x	x	x	x	x
0.81	x	x	x	x	x	x	x	x

8.2 ANOVA

Data were analysed using ANOVA (analysis of variance) with replicates, PPD level (high, medium and low), cultivars within PPD levels (C(LEV)) and cultivars as sources of variation.

The Analysis of Variance table is composed of the following columns:

Source: the source of variation

DF: the degrees of freedom

SS: the sum of squares

MS: the mean square

F: the F statistic

Pr>F: the p-value

The variables considered for the analysis were the measurements of secondary metabolites or enzymatic activity at each day during the time course or the natural logarithm of the area under the curve (AUTC).

8.2.1 ANOVA for Bath samples group

ESCULIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	25.439623	3.1799529	1.52	0.2485
REP	2	6.5000481	3.2500241	1.55	0.2519
LEVEL	2	4.1686684	2.0843342	0.56	0.6082
C(LEV)	4	14.770906	3.6927266	1.76	0.2015
CULT	6	18.939575	3.1565958	1.51	0.257
Error	12	25.162007	2.0968339		
Total	20	50.60163			

R-Square	C.V.	R-MSE	Mean
0.502743	118.7059	1.44804	1.2198591

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	529.53059	66.191323	1.1	0.4251
REP	2	101.51493	50.757465	0.84	0.454
LEVEL	2	75.605437	37.802718	0.43	0.6779
C(LEV)	4	352.41022	88.102555	1.47	0.2731
CULT	6	428.01566	71.335943	1.19	0.3758
Error	12	721.64467	60.137056		
Total	20	1251.1753			

R-Square	C.V.	R-MSE	Mean
0.423227	123.5231	7.75481	6.2780252

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	381.27075	47.658844	1.84	0.1643
REP	2	85.480777	42.740389	1.65	0.2327
LEVEL	2	55.792857	27.896428	0.46	0.6583
C(LEV)	4	239.99712	59.99928	2.32	0.1166
CULT	6	295.78998	49.298329	1.9	0.1613
Error	12	310.72126	25.893438		
Total	20	691.99201			

R-Square	C.V.	R-MSE	Mean
0.550976	149.6901	5.08856	3.3993967

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	5025.867	628.23338	0.93	0.5291
REP	2	291.62626	145.81313	0.21	0.8097
LEVEL	2	1942.9258	971.46289	1.39	0.3476
C(LEV)	4	2791.315	697.82875	1.03	0.432
CULT	6	4734.2408	789.04013	1.16	0.3866
Error	12	8145.1834	678.76529		
Total	20	13171.05			

R-Square	C.V.	R-MSE	Mean
0.381584	142.439	26.0531	18.290726

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	2233.2593	279.15742	0.89	0.5545
REP	2	402.30033	201.15017	0.64	0.5449
LEVEL	2	343.05871	171.52936	0.46	0.6604
C(LEV)	4	1487.9003	371.97507	1.18	0.3675
CULT	6	1830.959	305.15983	0.97	0.4852
Error	12	3777.6895	314.80746		
Total	20	6010.9488			

R-Square	C.V.	R-MSE	Mean
0.371532	207.7916	17.7428	8.5387538

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	8678.5618	1084.8202	1.12	0.4141
REP	2	1320.6016	660.30082	0.68	0.524
LEVEL	2	1760.885	880.44251	0.63	0.5786
C(LEV)	4	5597.0751	1399.2688	1.45	0.2785
CULT	6	7357.9601	1226.3267	1.27	0.3412
Error	12	11610.681	967.55677		
Total	20	20289.243			

R-Square	C.V.	R-MSE	Mean
0.427742	180.3491	31.1056	17.247423

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	353.67579	44.209474	1.36	0.3026
REP	2	53.264915	26.632457	0.82	0.4629
LEVEL	2	33.909951	16.954976	0.25	0.787
C(LEV)	4	266.50092	66.625231	2.06	0.1501
CULT	6	300.41087	50.068479	1.55	0.2451
Error	12	388.83018	32.402515		
Total	20	742.50597			

R-Square	C.V.	R-MSE	Mean
0.476327	109.9274	5.69232	5.1782529

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	21245.773	2655.7216	0.98	0.4925
REP	2	4191.0356	2095.5178	0.78	0.482
LEVEL	2	1901.9616	950.98079	0.25	0.7894
C(LEV)	4	15152.776	3788.1939	1.4	0.2913
CULT	6	17054.737	2842.4562	1.05	0.4401
Error	12	32398.219	2699.8516		
Total	20	53643.992			

R-Square	C.V.	R-MSE	Mean
0.396051	271.1996	51.9601	19.159355

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	9.5995276	1.1999409	1.11	0.4206
REP	2	0.2413114	0.1206557	0.11	0.8954
LEVEL	2	4.5716774	2.2858387	1.91	0.2616
C(LEV)	4	4.7865387	1.1966347	1.11	0.398
CULT	6	9.3582161	1.5597027	1.44	0.2773
Error	12	12.984487	1.0820406		
Total	20	22.584015			

R-Square	C.V.	R-MSE	Mean
0.425059	28.29154	1.04021	3.6767593

8.2.1 ANOVA for Bath samples group

ESCULETIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	807.21512	100.90189	0.62	0.7436
REP	2	153.55452	76.77726	0.47	0.6331
LEVEL	2	56.12501	28.062505	0.19	0.8356
C(LEV)	4	597.53559	149.3839	0.92	0.4818
CULT	6	653.6606	108.94343	0.67	0.6735
Error	12	1939.8812	161.65677		
Total	20	2747.0964			

R-Square	C.V.	R-MSE	Mean
0.293843	195.0001	12.7144	6.5202171

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	3739.1064	467.3883	0.93	0.526
REP	2	1386.3335	693.16675	1.38	0.2888
LEVEL	2	873.91989	436.95994	1.18	0.3951
C(LEV)	4	1478.853	369.71326	0.74	0.585
CULT	6	2352.7729	392.12882	0.78	0.6009
Error	12	6028.4744	502.37287		
Total	20	9767.5809			

R-Square	C.V.	R-MSE	Mean
0.382808	207.6337	22.4137	10.794817

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	1822.9247	227.86559	0.88	0.558
REP	2	695.1916	347.5958	1.34	0.2972
LEVEL	2	482.95114	241.47557	1.5	0.3269
C(LEV)	4	644.78199	161.1955	0.62	0.6545
CULT	6	1127.7331	187.95552	0.73	0.6367
Error	12	3101.4619	258.45516		
Total	20	4924.3867			

R-Square	C.V.	R-MSE	Mean
0.370183	168.3528	16.0765	9.5493186

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	1715.321	214.41512	0.77	0.635
REP	2	347.34223	173.67111	0.62	0.552
LEVEL	2	1277.4438	638.72188	28.22	0.0044
C(LEV)	4	90.535009	22.633752	0.08	0.9866
CULT	6	1367.9788	227.99646	0.82	0.5753
Error	12	3336.5863	278.04886		
Total	20	5051.9073			

R-Square	C.V.	R-MSE	Mean
0.339539	219.0486	16.6748	7.6123719

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	6.1316659	0.7664582	1.01	0.4787
REP	2	0.3220369	0.1610185	0.21	0.8124
LEVEL	2	3.7645918	1.8822959	3.68	0.1239
C(LEV)	4	2.0450372	0.5112593	0.67	0.6243
CULT	6	5.8096289	0.9682715	1.27	0.3395
Error	12	9.1378634	0.7614886		
Total	20	15.269529			

R-Square	C.V.	R-MSE	Mean
0.401562	22.74423	0.87263	3.8367234

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	4664.943	583.11787	1.14	0.4065
REP	2	631.13931	315.56965	0.61	0.557
LEVEL	2	1398.2016	699.10078	1.06	0.4269
C(LEV)	4	2635.6021	658.90053	1.28	0.3302
CULT	6	4033.8037	672.30061	1.31	0.3245
Error	12	6160.873	513.40608		
Total	20	10825.816			

R-Square	C.V.	R-MSE	Mean
0.430909	163.3	22.6585	13.87536

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	3338.5211	417.31514	0.78	0.6289
REP	2	279.91162	139.95581	0.26	0.7742
LEVEL	2	636.16887	318.08443	0.53	0.6273
C(LEV)	4	2422.4406	605.61016	1.13	0.3875
CULT	6	3058.6095	509.76825	0.95	0.4949
Error	12	6423.5334	535.29445		
Total	20	9762.0545			

R-Square	C.V.	R-MSE	Mean
0.34199	166.7873	23.1364	13.871819

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	1012.4943	126.56178	1.01	0.4754
REP	2	150.92408	75.462042	0.6	0.5627
LEVEL	2	480.11245	240.05622	2.52	0.196
C(LEV)	4	381.45774	95.364435	0.76	0.5694
CULT	6	861.57019	143.59503	1.15	0.3932
Error	12	1500.6121	125.05101		
Total	20	2513.1063			

R-Square	C.V.	R-MSE	Mean
0.402886	105.5085	11.1826	10.59879

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	292.91904	36.61488	0.75	0.6501
REP	2	15.323836	7.6619181	0.16	0.8564
LEVEL	2	110.40877	55.204386	1.32	0.3627
C(LEV)	4	167.18643	41.796608	0.86	0.5169
CULT	6	277.5952	46.265867	0.95	0.4973
Error	12	585.61769	48.801474		
Total	20	878.53673			

R-Square	C.V.	R-MSE	Mean
0.333417	106.8605	6.98581	6.5373143

8.2.1 ANOVA for Bath samples group

SCOPOLIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	1043.6956	130.46195	2.65	0.0627
REP	2	1.6358427	0.8179214	0.02	0.9836
LEVEL	2	160.78164	80.390822	0.36	0.7152
C(LEV)	4	881.27808	220.31952	4.47	0.0193
CULT	6	1042.0597	173.67662	3.52	0.0302
Error	12	591.63946	49.303288		
Total	20	1635.335			

R-Square	C.V.	R-MSE	Mean
0.638215	90.17512	7.02163	7.78666

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	35908.494	4488.5617	2.68	0.0604
REP	2	10610.257	5305.1284	3.17	0.0787
LEVEL	2	3203.5421	1601.7711	0.29	0.7628
C(LEV)	4	22094.695	5523.6737	3.3	0.0484
CULT	6	25298.237	4216.3728	2.52	0.0819
Error	12	20105.934	1675.4945		
Total	20	56014.428			

R-Square	C.V.	R-MSE	Mean
0.641058	88.5632	40.9328	46.218749

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	533.73363	66.716703	0.68	0.7041
REP	2	19.91877	9.959385	0.1	0.9046
LEVEL	2	24.595532	12.297766	0.1	0.9066
C(LEV)	4	489.21932	122.30483	1.24	0.3452
CULT	6	513.81486	85.635809	0.87	0.5444
Error	12	1182.3949	98.532906		
Total	20	1716.1285			

R-Square	C.V.	R-MSE	Mean
0.31101	101.4457	9.92637	9.7849152

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	69386.768	8673.346	1.49	0.2579
REP	2	471.64245	235.82122	0.04	0.9605
LEVEL	2	1024.2481	512.12403	0.03	0.9705
C(LEV)	4	67890.878	16972.719	2.91	0.0675
CULT	6	68915.126	11485.854	1.97	0.1495
Error	12	69945.564	5828.797		
Total	20	139332.33			

R-Square	C.V.	R-MSE	Mean
0.497995	139.8795	76.3466	54.58022

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	12498.94	1562.3676	1.46	0.2678
REP	2	803.89334	401.94667	0.38	0.6949
LEVEL	2	2089.8678	1044.9339	0.44	0.6745
C(LEV)	4	9605.1793	2401.2948	2.24	0.1253
CULT	6	11695.047	1949.1745	1.82	0.1777
Error	12	12851.77	1070.9808		
Total	20	25350.71			

R-Square	C.V.	R-MSE	Mean
0.493041	92.99315	32.7258	35.19167

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	33557.838	4194.7298	3.23	0.0334
REP	2	425.22623	212.61311	0.16	0.851
LEVEL	2	27879.661	13939.831	10.61	0.0251
C(LEV)	4	5252.9509	1313.2377	1.01	0.4403
CULT	6	33132.612	5522.102	4.25	0.0159
Error	12	15604.013	1300.3344		
Total	20	49161.851			

R-Square	C.V.	R-MSE	Mean
0.682599	86.76615	36.0601	41.560158

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	15146.359	1893.2949	0.81	0.6084
REP	2	3871.8006	1935.9003	0.83	0.461
LEVEL	2	4854.3078	2427.1539	1.51	0.3243
C(LEV)	4	6420.2504	1605.0626	0.69	0.6156
CULT	6	11274.558	1879.093	0.8	0.5866
Error	12	28103.139	2341.9282		
Total	20	43249.497			

R-Square	C.V.	R-MSE	Mean
0.350209	133.3409	48.3935	36.293045

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	34030.073	4253.7591	1.02	0.4682
REP	2	7605.8145	3802.9073	0.92	0.4265
LEVEL	2	299.84189	149.92094	0.02	0.9774
C(LEV)	4	26124.417	6531.1042	1.57	0.2443
CULT	6	26424.259	4404.0431	1.06	0.4362
Error	12	49835.432	4152.9527		
Total	20	83865.505			

R-Square	C.V.	R-MSE	Mean
0.40577	134.0707	64.4434	48.066722

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	7.6637965	0.9579746	1.88	0.1573
REP	2	0.2176108	0.1088054	0.21	0.8111
LEVEL	2	0.6070496	0.3035248	0.18	0.8436
C(LEV)	4	6.8391361	1.709784	3.35	0.0464
CULT	6	7.4461857	1.241031	2.43	0.0899
Error	12	6.1299762	0.5108314		
Total	20	13.793773			

R-Square	C.V.	R-MSE	Mean
0.555598	13.71326	0.71472	5.2119225

8.2.1 ANOVA for Bath samples group

SCOPOLETIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	2964.1423	370.51778	0.39	0.9028
REP	2	47.667646	23.833823	0.03	0.975
LEVEL	2	25.155204	12.577602	0.02	0.9828
C(LEV)	4	2891.3194	722.82985	0.77	0.5648
CULT	6	2916.4746	486.0791	0.52	0.7841
Error	12	11257.253	938.1044		
Total	20	14221.395			

R-Square	C.V.	R-MSE	Mean
0.208428	114.8182	30.6285	26.675645

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	20757.217	2594.6521	1.41	0.2855
REP	2	1169.0863	584.54317	0.32	0.7339
LEVEL	2	3048.931	1524.4655	0.37	0.7129
C(LEV)	4	16539.2	4134.8	2.25	0.1248
CULT	6	19588.131	3264.6885	1.77	0.1875
Error	12	22091.26	1840.9383		
Total	20	42848.477			

R-Square	C.V.	R-MSE	Mean
0.484433	91.00707	42.9062	47.145959

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	15631.26	1953.9075	0.44	0.8719
REP	2	1939.0249	969.51243	0.22	0.8053
LEVEL	2	4383.2521	2191.6261	0.94	0.4622
C(LEV)	4	9308.983	2327.2458	0.53	0.7166
CULT	6	13692.235	2282.0392	0.52	0.7834
Error	12	52750.306	4395.8589		
Total	20	68381.566			

R-Square	C.V.	R-MSE	Mean
0.228589	124.0304	66.3013	53.455678

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	7723.8717	965.48396	2.72	0.0575
REP	2	1446.341	723.17048	2.04	0.1728
LEVEL	2	1730.9537	865.47684	0.76	0.5246
C(LEV)	4	4546.577	1136.6443	3.2	0.0524
CULT	6	6277.5307	1046.2551	2.95	0.0524
Error	12	4255.8043	354.65035		
Total	20	11979.676			

R-Square	C.V.	R-MSE	Mean
0.644748	78.43746	18.8322	24.009144

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	2.6321722	0.3290215	1.33	0.3146
REP	2	0.1707993	0.0853996	0.35	0.7141
LEVEL	2	0.303792	0.151896	0.28	0.7684
C(LEV)	4	2.1575809	0.5393952	2.19	0.132
CULT	6	2.4613729	0.4102288	1.66	0.2131
Error	12	2.9585384	0.2465449		
Total	20	5.5907106			

R-Square	C.V.	R-MSE	Mean
0.470812	8.893221	0.49653	5.5832735

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	4185.1348	523.14184	0.92	0.5343
REP	2	560.0218	280.0109	0.49	0.6237
LEVEL	2	1040.7237	520.36184	0.81	0.5082
C(LEV)	4	2584.3893	646.09732	1.13	0.3868
CULT	6	3625.113	604.18549	1.06	0.4366
Error	12	6842.1217	570.17681		
Total	20	11027.256			

R-Square	C.V.	R-MSE	Mean
0.379526	86.60833	23.8784	27.570529

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	10355.059	1294.3824	1.26	0.3469
REP	2	908.48257	454.24128	0.44	0.6529
LEVEL	2	5349.8919	2674.9459	2.61	0.1881
C(LEV)	4	4096.685	1024.1713	1	0.4468
CULT	6	9446.5769	1574.4295	1.53	0.2492
Error	12	12338.106	1028.1755		
Total	20	22693.166			

R-Square	C.V.	R-MSE	Mean
0.456307	78.94449	32.0652	40.617369

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	41674.117	5209.2646	1.74	0.1855
REP	2	1946.8728	973.43638	0.33	0.728
LEVEL	2	17691.237	8845.6187	1.61	0.3077
C(LEV)	4	22036.007	5509.0017	1.85	0.1851
CULT	6	39727.244	6621.2074	2.22	0.1132
Error	12	35826.205	2985.5171		
Total	20	77500.322			

R-Square	C.V.	R-MSE	Mean
0.537728	89.00675	54.6399	61.388476

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	34324.111	4290.5138	1.81	0.1705
REP	2	7555.4339	3777.7169	1.59	0.2431
LEVEL	2	3278.0952	1639.0476	0.28	0.7701
C(LEV)	4	23490.582	5872.6454	2.48	0.1001
CULT	6	26768.677	4461.4461	1.88	0.1651
Error	12	28421.864	2368.4887		
Total	20	62745.975			

R-Square	C.V.	R-MSE	Mean
0.547033	107.1636	48.6671	45.41387

8.2.1 ANOVA for Bath samples group

(+)-GALLOCATECHIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	291.19261	36.399076	1.56	0.2358
REP	2	44.752875	22.376437	0.96	0.4114
LEVEL	2	21.909626	10.954813	0.2	0.8301
C(LEV)	4	224.53011	56.132527	2.4	0.1076
CULT	6	246.43974	41.073289	1.76	0.191
Error	12	280.45652	23.371377		
Total	20	571.64913			

R-Square	C.V.	R-MSE	Mean
0.50939	209.6226	4.8344	2.3062381

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	269.38049	33.672561	1.85	0.1633
REP	2	38.369017	19.184508	1.05	0.3795
LEVEL	2	102.1248	51.062398	1.58	0.3113
C(LEV)	4	128.88667	32.221668	1.77	0.2005
CULT	6	231.01147	38.501912	2.11	0.1276
Error	12	218.9356	18.244633		
Total	20	488.31609			

R-Square	C.V.	R-MSE	Mean
0.551652	189.6363	4.27137	2.2524029

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	7729.8224	966.2278	0.7	0.6888
REP	2	1381.0415	690.52074	0.5	0.6194
LEVEL	2	1565.5171	782.75854	0.65	0.5676
C(LEV)	4	4783.2638	1195.816	0.86	0.5132
CULT	6	6348.7809	1058.1302	0.76	0.6119
Error	12	16619.48	1384.9566		
Total	20	24349.302			

R-Square	C.V.	R-MSE	Mean
0.317456	319.1786	37.215	11.659617

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	9031.2953	1128.9119	0.95	0.5153
REP	2	4693.6598	2346.8299	1.97	0.1822
LEVEL	2	572.98647	286.49324	0.3	0.7533
C(LEV)	4	3764.6491	941.16226	0.79	0.5539
CULT	6	4337.6355	722.93925	0.61	0.7211
Error	12	14304.662	1192.0551		
Total	20	23335.957			

R-Square	C.V.	R-MSE	Mean
0.387012	171.9961	34.5262	20.073796

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	30.301306	3.7876633	2.29	0.0951
REP	2	5.6743704	2.8371852	1.71	0.2216
LEVEL	2	8.1920752	4.0960376	1	0.4454
C(LEV)	4	16.434861	4.1087152	2.48	0.0999
CULT	6	24.626936	4.1044894	2.48	0.0853
Error	12	19.87375	1.6561458		
Total	20	50.175056			

R-Square	C.V.	R-MSE	Mean
0.603912	24.47853	1.28691	5.2573152

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	71032.549	8879.0686	0.96	0.5073
REP	2	16624.405	8312.2024	0.9	0.4329
LEVEL	2	21360.107	10680.053	1.29	0.3689
C(LEV)	4	33048.037	8262.0093	0.89	0.4976
CULT	6	54408.144	9068.024	0.98	0.4791
Error	12	111009.5	9250.7917		
Total	20	182042.05			

R-Square	C.V.	R-MSE	Mean
0.390199	190.481	96.181	50.493771

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	68897.147	8612.1434	0.77	0.6381
REP	2	14470.677	7235.3386	0.64	0.5423
LEVEL	2	6237.8104	3118.9052	0.26	0.7839
C(LEV)	4	48188.66	12047.165	1.07	0.4122
CULT	6	54426.47	9071.0784	0.81	0.5832
Error	12	134782.38	11231.865		
Total	20	203679.53			

R-Square	C.V.	R-MSE	Mean
0.338263	137.8758	105.98	76.866612

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	581062.55	72632.819	1.82	0.1686
REP	2	184976.14	92488.07	2.32	0.1409
LEVEL	2	216382.58	108191.29	2.41	0.2058
C(LEV)	4	179703.84	44925.959	1.13	0.3898
CULT	6	396086.41	66014.402	1.65	0.2155
Error	12	478842.47	39903.539		
Total	20	1059905			

R-Square	C.V.	R-MSE	Mean
0.548221	133.5187	199.759	149.61101

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	566978.74	70872.342	1.71	0.1936
REP	2	63940.723	31970.361	0.77	0.4838
LEVEL	2	284553.92	142276.96	2.6	0.1886
C(LEV)	4	218484.09	54621.022	1.32	0.3182
CULT	6	503038.01	83839.669	2.02	0.1406
Error	12	497010.94	41417.579		
Total	20	1063989.7			

R-Square	C.V.	R-MSE	Mean
0.53288	137.272	203.513	148.25535

8.2.1 ANOVA for Bath samples group

(+)-CATECHIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	193.89738	24.237172	0.88	0.557
REP	2	44.070408	22.035204	0.8	0.4707
LEVEL	2	46.17289	23.086445	0.89	0.4786
C(LEV)	4	103.65408	25.91352	0.94	0.4718
CULT	6	149.82697	24.971161	0.91	0.5197
Error	12	329.35433	27.446194		
Total	20	523.2517			

R-Square	C.V.	R-MSE	Mean
0.370562	161.3982	5.23891	3.2459543

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	2759.7104	344.96381	0.99	0.4867
REP	2	796.34487	398.17244	1.15	0.3502
LEVEL	2	471.55081	235.77541	0.63	0.5773
C(LEV)	4	1491.8148	372.95369	1.07	0.4117
CULT	6	1963.3656	327.2276	0.94	0.5007
Error	12	4167.5201	347.29335		
Total	20	6927.2306			

R-Square	C.V.	R-MSE	Mean
0.398386	199.7428	18.6358	9.3299005

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	1437.5453	179.69316	0.61	0.751
REP	2	262.00107	131.00054	0.45	0.6493
LEVEL	2	293.93579	146.96789	0.67	0.5624
C(LEV)	4	881.60845	220.40211	0.75	0.5747
CULT	6	1175.5442	195.92404	0.67	0.6764
Error	12	3510.3156	292.5263		
Total	20	4947.8609			

R-Square	C.V.	R-MSE	Mean
0.290539	209.4491	17.1034	8.1658981

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	816.1421	102.01776	1.41	0.2868
REP	2	9.2304661	4.615233	0.06	0.9387
LEVEL	2	188.07676	94.038381	0.61	0.5882
C(LEV)	4	618.83487	154.70872	2.13	0.1393
CULT	6	806.91163	134.48527	1.85	0.1709
Error	12	870.70036	72.558363		
Total	20	1686.8425			

R-Square	C.V.	R-MSE	Mean
0.483828	129.8976	8.51812	6.5575667

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	4.5598808	0.5699851	0.65	0.7212
REP	2	0.8409539	0.420477	0.48	0.6286
LEVEL	2	1.6519843	0.8259921	1.6	0.3089
C(LEV)	4	2.0669426	0.5167357	0.59	0.6743
CULT	6	3.7189269	0.6198212	0.71	0.6475
Error	12	10.455079	0.8712566		
Total	20	15.01496			

R-Square	C.V.	R-MSE	Mean
0.303689	28.26971	0.93341	3.301807

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	5728.1529	716.01911	0.82	0.5983
REP	2	1593.3147	796.65737	0.92	0.4266
LEVEL	2	3594.5802	1797.2901	13.31	0.0171
C(LEV)	4	540.2579	135.06448	0.16	0.9569
CULT	6	4134.8381	689.13969	0.79	0.5935
Error	12	10442.953	870.2461		
Total	20	16171.106			

R-Square	C.V.	R-MSE	Mean
0.354221	209.8081	29.4999	14.060433

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	11130.542	1391.3178	0.86	0.5713
REP	2	657.56932	328.78466	0.2	0.8185
LEVEL	2	1289.1779	644.58895	0.28	0.769
C(LEV)	4	9183.7953	2295.9488	1.42	0.2855
CULT	6	10472.973	1745.4955	1.08	0.4256
Error	12	19369.811	1614.1509		
Total	20	30500.354			

R-Square	C.V.	R-MSE	Mean
0.364932	164.7421	40.1765	24.387518

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	50976.925	6372.1156	1	0.484
REP	2	4903.1862	2451.5931	0.38	0.6893
LEVEL	2	21524.471	10762.236	1.75	0.2839
C(LEV)	4	24549.268	6137.3169	0.96	0.4636
CULT	6	46073.739	7678.9565	1.2	0.3688
Error	12	76646.532	6387.211		
Total	20	127623.46			

R-Square	C.V.	R-MSE	Mean
0.399432	150.071	79.92	53.254815

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	18800.369	2350.0462	1.14	0.404
REP	2	2812.9285	1406.4642	0.68	0.5239
LEVEL	2	9800.8709	4900.4354	3.17	0.1497
C(LEV)	4	6186.57	1546.6425	0.75	0.5764
CULT	6	15987.441	2664.5735	1.29	0.3309
Error	12	24726.497	2060.5414		
Total	20	43526.867			

R-Square	C.V.	R-MSE	Mean
0.431926	141.3503	45.3932	32.113962

8.2.1 ANOVA for Bath samples group

(+)-CATECHIN GALLATE

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	8	110.58213	13.822766	1.72	0.1909
REP	2	2.2587531	1.1293766	0.14	0.8702
LEVEL	2	40.046338	20.023169	1.17	0.3973
C(LEV)	4	68.277039	17.06926	2.13	0.1401
CULT	6	108.32338	18.053896	2.25	0.1094
Error	12	96.328873	8.0274061		
Total	20	206.911			

R-Square	C.V.	R-MSE	Mean
0.534443	118.9811	2.83327	2.3812752

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	8	269.92978	33.741223	0.97	0.5011
REP	2	83.19869	41.599345	1.2	0.3361
LEVEL	2	131.68616	65.843078	4.78	0.0869
C(LEV)	4	55.044936	13.761234	0.4	0.8081
CULT	6	186.73109	31.121848	0.89	0.5289
Error	12	417.54315	34.795262		
Total	20	687.47293			

R-Square	C.V.	R-MSE	Mean
0.392641	126.0833	5.89875	4.6784538

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	8	55.515286	6.9394107	0.92	0.5355
REP	2	8.5644805	4.2822403	0.57	0.5827
LEVEL	2	11.468638	5.7343192	0.65	0.5711
C(LEV)	4	35.482167	8.8705417	1.17	0.3719
CULT	6	46.950805	7.8251342	1.03	0.4507
Error	12	90.939564	7.578297		
Total	20	146.45485			

R-Square	C.V.	R-MSE	Mean
0.379061	128.9705	2.75287	2.1344957

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	8	680.9327	85.116588	1.13	0.41
REP	2	208.67365	104.33682	1.38	0.2878
LEVEL	2	113.57025	56.785123	0.63	0.5769
C(LEV)	4	358.68881	89.672203	1.19	0.3645
CULT	6	472.25906	78.709843	1.04	0.4447
Error	12	904.6622	75.388516		
Total	20	1585.5949			

R-Square	C.V.	R-MSE	Mean
0.429449	159.7778	8.68266	5.4342052

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	8	490.40324	61.300405	1.33	0.3151
REP	2	161.60623	80.803116	1.76	0.2141
LEVEL	2	80.799173	40.399586	0.65	0.5689
C(LEV)	4	247.99784	61.999459	1.35	0.3085
CULT	6	328.79701	54.799501	1.19	0.3734
Error	12	551.76813	45.980678		
Total	20	1042.1714			

R-Square	C.V.	R-MSE	Mean
0.470559	189.908	6.78091	3.5706262

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	8	1959.4878	244.93597	0.84	0.5855
REP	2	632.55881	316.27941	1.09	0.3685
LEVEL	2	396.52073	198.26036	0.85	0.4916
C(LEV)	4	930.40823	232.60206	0.8	0.5486
CULT	6	1326.929	221.15483	0.76	0.6149
Error	12	3494.4462	291.20385		
Total	20	5453.9339			

R-Square	C.V.	R-MSE	Mean
0.35928	187.0503	17.0647	9.1230514

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	8	106.44358	13.305447	1.31	0.3249
REP	2	8.2548101	4.1274051	0.41	0.675
LEVEL	2	35.868253	17.934126	1.15	0.4028
C(LEV)	4	62.320514	15.580128	1.53	0.2544
CULT	6	98.188767	16.364794	1.61	0.2269
Error	12	121.92839	10.160699		
Total	20	228.37196			

R-Square	C.V.	R-MSE	Mean
0.466097	129.3875	3.18759	2.4635967

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	8	983.69533	122.96192	0.39	0.9054
REP	2	204.76067	102.38033	0.33	0.7285
LEVEL	2	593.76287	296.88143	6.41	0.0565
C(LEV)	4	185.17179	46.292948	0.15	0.9608
CULT	6	778.93466	129.82244	0.41	0.857
Error	12	3777.6926	314.80772		
Total	20	4761.388			

R-Square	C.V.	R-MSE	Mean
0.206598	143.2233	17.7428	12.388223

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	8	10.192281	1.2740351	0.83	0.5902
REP	2	0.6450941	0.3225471	0.21	0.8125
LEVEL	2	3.4074392	1.7037196	1.11	0.4136
C(LEV)	4	6.1397478	1.534937	1.01	0.4425
CULT	6	9.547187	1.5911978	1.04	0.4457
Error	12	18.32435	1.5270292		
Total	20	28.516631			

R-Square	C.V.	R-MSE	Mean
0.357415	28.10979	1.23573	4.396085

8.2.2 ANOVA for June 1999 samples group

POX

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	2.364E-05	3.38E-06	1.92	0.1695
REP	2	1.76E-06	8.8E-07	0.5	0.6209
LEVEL	2	1.758E-05	8.79E-06	6.15	0.0868
C(LEV)	3	4.29E-06	1.43E-06	0.81	0.5164
CULT	5	2.187E-05	4.37E-06	2.48	0.1038
Error	10	1.763E-05	1.76E-06		
Total	17	4.127E-05			

R-Square	C.V.	R-MSE	Mean
0.572761	83.8598	0.00133	0.0015833

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	6.121E-05	8.74E-06	13.39	0.0002
REP	2	3.2E-07	1.6E-07	0.25	0.7847
LEVEL	2	1.687E-05	8.44E-06	0.58	0.6146
C(LEV)	3	4.401E-05	1.467E-05	22.47	0.0001
CULT	5	6.088E-05	1.218E-05	18.65	0.0001
Error	10	6.53E-06	6.5E-07		
Total	17	6.774E-05			

R-Square	C.V.	R-MSE	Mean
0.903613	20.57182	0.00081	0.0039278

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	5.156E-05	7.37E-06	5.22	0.0099
REP	2	1.2E-07	6E-08	0.04	0.9574
LEVEL	2	1.906E-05	9.53E-06	0.88	0.4993
C(LEV)	3	3.237E-05	1.079E-05	7.65	0.006
CULT	5	5.143E-05	1.029E-05	7.29	0.004
Error	10	0.0000141	1.41E-06		
Total	17	6.566E-05			

R-Square	C.V.	R-MSE	Mean
0.785207	64.77679	0.00119	0.0018333

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	0.0003039	4.341E-05	13.19	0.0003
REP	2	3.2E-07	1.6E-07	0.05	0.9523
LEVEL	2	6.096E-05	3.048E-05	0.38	0.7145
C(LEV)	3	0.0002426	8.087E-05	24.58	0.0001
CULT	5	0.0003036	6.072E-05	18.45	0.0001
Error	10	0.0000329	3.29E-06		
Total	17	0.0003368			

R-Square	C.V.	R-MSE	Mean
0.902307	31.00731	0.00181	0.00585

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	3.491E-05	4.99E-06	3.63	0.0326
REP	2	1.34E-06	6.7E-07	0.49	0.6287
LEVEL	2	1.419E-05	0.0000071	1.1	0.4386
C(LEV)	3	1.938E-05	6.46E-06	4.7	0.027
CULT	5	3.357E-05	6.71E-06	4.88	0.0161
Error	10	1.376E-05	1.38E-06		
Total	17	4.866E-05			

R-Square	C.V.	R-MSE	Mean
0.717329	66.59664	0.00117	0.0017611

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	0.000148	2.115E-05	3.81	0.028
REP	2	5.59E-06	2.79E-06	0.5	0.619
LEVEL	2	1.156E-05	5.78E-06	0.13	0.8807
C(LEV)	3	0.0001309	4.363E-05	7.86	0.0055
CULT	5	0.0001425	2.849E-05	5.13	0.0137
Error	10	5.552E-05	5.55E-06		
Total	17	0.0002036			

R-Square	C.V.	R-MSE	Mean
0.727271	35.69996	0.00236	0.0066

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	4.022E-05	5.75E-06	7.45	0.0027
REP	2	1.29E-06	6.4E-07	0.83	0.462
LEVEL	2	1.352E-05	6.76E-06	0.8	0.5274
C(LEV)	3	2.541E-05	8.47E-06	10.98	0.0017
CULT	5	3.894E-05	7.79E-06	10.1	0.0012
Error	10	7.71E-06	7.7E-07		
Total	17	4.794E-05			

R-Square	C.V.	R-MSE	Mean
0.839115	41.27276	0.00088	0.0021278

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	0.0003383	4.833E-05	2.02	0.1508
REP	2	0.0000199	9.95E-06	0.42	0.6704
LEVEL	2	0.0001224	6.122E-05	0.94	0.4829
C(LEV)	3	0.000196	6.534E-05	2.73	0.0995
CULT	5	0.0003184	6.369E-05	2.66	0.0878
Error	10	0.000239	0.0000239		
Total	17	0.0005773			

R-Square	C.V.	R-MSE	Mean
0.586039	56.12048	0.00489	0.0087111

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	0.0031692	0.0004527	26.59	0.0001
REP	2	0.0000015	7.5E-07	0.04	0.9572
LEVEL	2	0.0005252	0.0002626	0.3	0.7619
C(LEV)	3	0.0026425	0.0008808	51.72	0.0001
CULT	5	0.0031677	0.0006335	37.2	0.0001
Error	10	0.0001703	1.703E-05		
Total	17	0.0033395			

R-Square	C.V.	R-MSE	Mean
0.949004	15.40417	0.00413	0.0267897

8.2.2 ANOVA for June 1999 samples group

PPO

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	5.79E-06	8.3E-07	1.6	0.2418
REP	2	1.05E-06	5.3E-07	1.02	0.3959
LEVEL	2	1.46E-06	7.3E-07	0.67	0.5742
C(LEV)	3	3.27E-06	1.09E-06	2.11	0.1629
CULT	5	4.73E-06	9.5E-07	1.83	0.1946
Error	10	5.17E-06	5.2E-07		
Total	17	1.096E-05			

R-Square	C.V.	R-MSE	Mean
0.527981	165.9828	0.00072	0.0004333

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	8.278E-05	1.183E-05	2.3	0.122
REP	2	8.32E-06	4.16E-06	0.81	0.4755
LEVEL	2	1.791E-05	8.96E-06	0.61	0.5997
C(LEV)	3	0.0000441	0.0000147	2.86	0.097
CULT	5	7.324E-05	1.465E-05	2.85	0.0821
Error	9	4.633E-05	5.15E-06		
Total	16	0.0001291			

R-Square	C.V.	R-MSE	Mean
0.641172	110.8303	0.00227	0.0020471

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	6.257E-05	8.94E-06	4.23	0.0201
REP	2	5.3E-07	2.7E-07	0.13	0.8826
LEVEL	2	0.0000182	0.0000091	0.62	0.5939
C(LEV)	3	4.383E-05	1.461E-05	6.91	0.0084
CULT	5	6.203E-05	1.241E-05	5.87	0.0087
Error	10	2.113E-05	2.11E-06		
Total	17	0.0000837			

R-Squa	C.V.	R-MSE	Mean
0.75	97.63607	0.0014537	0.0014889

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	0.0004781	6.831E-05	5.71	0.0072
REP	2	1.472E-05	7.36E-06	0.62	0.5595
LEVEL	2	0.0002147	0.0001074	1.3	0.3931
C(LEV)	3	0.0002487	0.0000829	6.93	0.0083
CULT	5	0.0004634	9.268E-05	7.75	0.0032
Error	10	0.0001195	1.195E-05		
Total	17	0.0005977			

R-Square	C.V.	R-MSE	Mean
0.799988	109.1843	0.00346	0.0031667

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	5.374E-05	7.68E-06	7.04	0.0033
REP	2	7.2E-07	3.6E-07	0.33	0.7253
LEVEL	2	1.284E-05	6.42E-06	0.48	0.6596
C(LEV)	3	4.017E-05	1.339E-05	12.28	0.0011
CULT	5	5.302E-05	0.0000106	9.73	0.0013
Error	10	0.0000109	1.09E-06		
Total	17	6.464E-05			

R-Square	C.V.	R-MSE	Mean
0.831335	73.70755	0.00104	0.0014167

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	0.0003675	0.0000525	2.74	0.0722
REP	2	2.515E-05	1.258E-05	0.66	0.5394
LEVEL	2	0.0001345	6.727E-05	0.97	0.473
C(LEV)	3	0.0002078	6.928E-05	3.62	0.0531
CULT	5	0.0003424	6.848E-05	3.58	0.0409
Error	10	0.0001914	1.914E-05		
Total	17	0.000559			

R-Square	C.V.	R-MSE	Mean
0.657523	148.595	0.00438	0.0029444

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	5.402E-05	7.72E-06	10.07	0.0008
REP	2	4.91E-06	2.46E-06	3.21	0.084
LEVEL	2	1.897E-05	9.48E-06	0.94	0.4808
C(LEV)	3	3.013E-05	1.005E-05	13.1	0.0008
CULT	5	0.0000491	9.82E-06	12.81	0.0004
Error	10	7.67E-06	7.7E-07		
Total	17	6.168E-05			

R-Square	C.V.	R-MSE	Mean
0.875726	64.32477	0.00088	0.0013611

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	0.0007636	0.0001091	8.06	0.0019
REP	2	0.0000601	3.005E-05	2.22	0.1592
LEVEL	2	0.0003258	0.0001629	1.29	0.3934
C(LEV)	3	0.0003777	0.0001259	9.3	0.0031
CULT	5	0.0007035	0.0001407	10.4	0.001
Error	10	0.0001353	1.353E-05		
Total	17	0.000899			

R-Square	C.V.	R-MSE	Mean
0.849457	75.84995	0.00368	0.00485

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	0.0066859	0.0009551	84.93	0.0001
REP	2	6.516E-05	3.258E-05	2.9	0.1018
LEVEL	2	0.0024544	0.0012272	0.88	0.4992
C(LEV)	3	0.0041663	0.0013888	123.49	0.0001
CULT	5	0.0066207	0.0013242	117.74	0.0001
Error	10	0.0001125	1.125E-05		
Total	17	0.0067984			

R-Square	C.V.	R-MSE	Mean
0.983457	22.98806	0.00335	0.0145883

8.2.2 ANOVA for June 1999 samples group

CAT

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	5.9E-07	8E-08	20.09	0.0001
REP	2	6E-08	3E-08	6.84	0.0134
LEVEL	2	2.4E-07	1.2E-07	1.23	0.4082
C(LEV)	3	0.0000003	0.0000001	23.29	0.0001
CULT	5	5.4E-07	1.1E-07	25.39	0.0001
Error	10	4E-08	0		
Total	17	6.4E-07			
		R-Square	C.V.	R-MSE	Mean
		0.933624	37.72953	6.5E-05	0.0001722

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	4.5E-07	6E-08	3.3	0.0431
REP	2	5E-08	3E-08	1.38	0.2958
LEVEL	2	3.1E-07	1.6E-07	5.58	0.0975
C(LEV)	3	8E-08	3E-08	1.44	0.2897
CULT	5	3.9E-07	8E-08	4.07	0.0282
Error	10	1.9E-07	2E-08		
Total	17	6.4E-07			
		R-Square	C.V.	R-MSE	Mean
		0.697917	59.59044	0.00014	0.0002333

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	1.5E-07	2E-08	4.12	0.0219
REP	2	1.2E-07	6E-08	11.6	0.0025
LEVEL	2	0	0	0.06	0.9439
C(LEV)	3	3E-08	1E-08	1.81	0.2092
CULT	5	3E-08	1E-08	1.13	0.4058
Error	10	5E-08	1E-08		
Total	17	0.0000002			
		R-Square	C.V.	R-MSE	Mean
		0.742466	52.03076	7.2E-05	0.0001389

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	3.3E-07	5E-08	5.04	0.0111
REP	2	1E-08	1E-08	0.78	0.4831
LEVEL	2	1.4E-07	7E-08	1.19	0.4159
C(LEV)	3	1.7E-07	6E-08	6.27	0.0115
CULT	5	3.1E-07	6E-08	6.75	0.0053
Error	10	9E-08	1E-08		
Total	17	4.2E-07			
		R-Square	C.V.	R-MSE	Mean
		0.779255	50.84068	9.6E-05	0.0001889

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	2.3E-07	3E-08	0.89	0.549
REP	2	2E-08	1E-08	0.32	0.7366
LEVEL	2	0.0000001	5E-08	1.5	0.3536
C(LEV)	3	0.0000001	3E-08	0.93	0.4612
CULT	5	2.1E-07	4E-08	1.12	0.4104
Error	10	3.7E-07	4E-08		
Total	17	0.0000006			
	R-Squa	C.V.	R-MSE		Mean
	0.38	96.17692	0.0001924		0.0002

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	1.5E-07	2E-08	3.06	0.0535
REP	2	2E-08	1E-08	1.67	0.2373
LEVEL	2	1E-08	0	0.13	0.8839
C(LEV)	3	1.2E-07	4E-08	5.56	0.0166
CULT	5	1.3E-07	3E-08	3.62	0.0396
Error	10	7E-08	1E-08		
Total	17	2.2E-07			
		R-Square	C.V.	R-MSE	Mean
		0.681818	41.833	8.4E-05	0.0002

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	0.0000004	6E-08	1.61	0.2382
REP	2	1.7E-07	9E-08	2.48	0.1338
LEVEL	2	1.4E-07	7E-08	2.59	0.222
C(LEV)	3	8E-08	3E-08	0.77	0.5352
CULT	5	2.2E-07	4E-08	1.26	0.3504
Error	10	3.5E-07	4E-08		
Total	17	7.5E-07			
		R-Square	C.V.	R-MSE	Mean
		0.530022	96.51901	0.00019	0.0001944

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	2.3E-07	3E-08	4.42	0.0174
REP	2	5E-08	3E-08	3.64	0.065
LEVEL	2	4E-08	2E-08	0.45	0.6747
C(LEV)	3	1.3E-07	4E-08	6.06	0.0128
CULT	5	1.7E-07	3E-08	4.73	0.0178
Error	10	7E-08	1E-08		
Total	17	0.0000003			
		R-Square	C.V.	R-MSE	Mean
		0.755556	51.38093	8.6E-05	0.0001667

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	0.0000085	1.21E-06	3.02	0.0557
REP	2	2.08E-06	1.04E-06	2.58	0.1249
LEVEL	2	2.56E-06	1.28E-06	0.99	0.4663
C(LEV)	3	3.86E-06	1.29E-06	3.2	0.0709
CULT	5	6.42E-06	1.28E-06	3.19	0.0558
Error	10	4.02E-06	0.0000004		
Total	17	1.252E-05			
		R-Square	C.V.	R-MSE	Mean
		0.678666	47.91334	0.00063	0.0013238

8.2.2 ANOVA for June 1999 samples group

PAL

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	1.32E-06	1.9E-07	1.43	0.292
REP	2	1.9E-07	9E-08	0.72	0.5124
LEVEL	2	5.9E-07	0.0000003	1.65	0.3291
C(LEV)	3	5.4E-07	1.8E-07	1.37	0.3083
CULT	5	1.13E-06	2.3E-07	1.72	0.2173
Error	10	1.31E-06	1.3E-07		
Total	17	2.63E-06			

R-Square	C.V.	R-MSE	Mean
0.500951	91.83703	0.00036	0.0003944

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	1.505E-05	2.15E-06	5.55	0.0079
REP	2	5.7E-07	2.9E-07	0.74	0.5015
LEVEL	2	6.01E-06	0.000003	1.07	0.4472
C(LEV)	3	8.46E-06	2.82E-06	7.28	0.0071
CULT	5	1.447E-05	2.89E-06	7.47	0.0037
Error	10	3.87E-06	3.9E-07		
Total	17	1.892E-05			

R-Square	C.V.	R-MSE	Mean
0.795278	88.90873	0.00062	0.0007

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	3.23E-06	4.6E-07	1.64	0.2312
REP	2	2.1E-07	1.1E-07	0.38	0.6931
LEVEL	2	1.29E-06	6.4E-07	1.12	0.4338
C(LEV)	3	1.73E-06	5.8E-07	2.04	0.1717
CULT	5	3.02E-06	0.0000006	2.14	0.1432
Error	10	2.82E-06	2.8E-07		
Total	17	6.05E-06			

R-Square	C.V.	R-MSE	Mean
0.534025	107.3795	0.00053	0.0004944

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	4.2E-07	6E-08	2.34	0.108
REP	2	1.5E-07	7E-08	2.85	0.1046
LEVEL	2	0.0000001	5E-08	0.87	0.5046
C(LEV)	3	1.8E-07	6E-08	2.25	0.1447
CULT	5	2.8E-07	6E-08	2.13	0.1442
Error	10	2.6E-07	3E-08		
Total	17	6.8E-07			

R-Square	C.V.	R-MSE	Mean
0.62083	44.55699	0.00016	0.0003611

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	0.0001044	1.491E-05	8.15	0.0019
REP	2	3.68E-06	1.84E-06	1.01	0.4001
LEVEL	2	3.955E-05	1.977E-05	0.97	0.4732
C(LEV)	3	6.115E-05	2.038E-05	11.14	0.0016
CULT	5	0.0001007	2.014E-05	11	0.0008
Error	10	0.0000183	1.83E-06		
Total	17	0.0001227			

R-Square	C.V.	R-MSE	Mean
0.850811	35.23846	0.00135	0.0038392

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	1.34E-06	1.9E-07	1.83	0.1861
REP	2	2.5E-07	1.3E-07	1.2	0.3416
LEVEL	2	4.8E-07	2.4E-07	1.19	0.4171
C(LEV)	3	6.1E-07	0.0000002	1.94	0.1874
CULT	5	1.09E-06	2.2E-07	2.08	0.1513
Error	10	1.04E-06	0.0000001		
Total	17	2.38E-06			

R-Square	C.V.	R-MSE	Mean
0.561625	60.56376	0.00032	0.0005333

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	1.07E-06	1.5E-07	3.14	0.0499
REP	2	0	0	0.05	0.9558
LEVEL	2	6.7E-07	3.4E-07	2.56	0.2245
C(LEV)	3	0.0000004	1.3E-07	2.69	0.1026
CULT	5	1.07E-06	2.1E-07	4.37	0.0227
Error	10	4.9E-07	5E-08		
Total	17	1.56E-06			

R-Square	C.V.	R-MSE	Mean
0.687167	41.03041	0.00022	0.0005389

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	3.41E-06	4.9E-07	2.29	0.1132
REP	2	1.9E-07	9E-08	0.44	0.6549
LEVEL	2	6.9E-07	3.4E-07	0.41	0.6978
C(LEV)	3	2.54E-06	8.5E-07	3.98	0.0419
CULT	5	3.22E-06	6.4E-07	3.03	0.0637
Error	10	2.13E-06	2.1E-07		
Total	17	5.54E-06			

R-Square	C.V.	R-MSE	Mean
0.616172	66.9248	0.00046	0.0006889

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	3.62E-06	5.2E-07	3.47	0.0372
REP	2	8.3E-07	4.2E-07	2.8	0.1085
LEVEL	2	9.1E-07	4.5E-07	0.72	0.554
C(LEV)	3	1.88E-06	6.3E-07	4.2	0.0364
CULT	5	2.79E-06	5.6E-07	3.74	0.0361
Error	10	1.49E-06	1.5E-07		
Total	17	5.12E-06			

R-Square	C.V.	R-MSE	Mean
0.708329	57.46506	0.00039	0.0006722

8.2.2 ANOVA for June 1999 samples group

GLUCANASE

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	2.370909	0.3387013	3.27	0.0508
REP	2	1.6625523	0.8312761	8.04	0.0099
LEVEL	2	0.2531052	0.1265526	1.23	0.4067
C(LEV)	3	0.3080298	0.1026766	0.99	0.4392
CULT	5	0.534769	0.1069538	1.03	0.4533
Error	9	0.9308943	0.1034327		
Total	16	3.3018034			

R-Square	C.V.	R-MSE	Mean
0.718065	100.2505	0.32161	0.3208059

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	1.4445797	0.2063685	4.42	0.0213
REP	2	1.0623549	0.5311775	11.38	0.0034
LEVEL	2	0.1744673	0.0872337	1.49	0.3551
C(LEV)	3	0.1755084	0.0585028	1.25	0.347
CULT	5	0.3479363	0.0695873	1.49	0.2835
Error	9	0.4199972	0.0466664		
Total	16	1.8645769			

R-Square	C.V.	R-MSE	Mean
0.774749	71.8235	0.21602	0.3007706

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	2.5815419	0.3687917	1.29	0.3449
REP	2	0.7737563	0.3868782	1.35	0.3018
LEVEL	2	0.4897997	0.2448998	0.56	0.6225
C(LEV)	3	1.3179859	0.4393286	1.54	0.2648
CULT	5	1.8077856	0.3615571	1.27	0.3503
Error	10	2.8575238	0.2857524		
Total	17	5.4390657			

R-Square	C.V.	R-MSE	Mean
0.47463	128.8575	0.53456	0.4148444

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	3.1947156	0.4563879	2.69	0.0844
REP	2	0.5769907	0.2884954	1.7	0.2365
LEVEL	2	1.1348567	0.5674283	1.19	0.4161
C(LEV)	3	1.429044	0.476348	2.81	0.1005
CULT	5	2.5963642	0.5192728	3.06	0.0692
Error	9	1.5276946	0.1697439		
Total	16	4.7224102			

R-Square	C.V.	R-MSE	Mean
0.676501	61.74404	0.412	0.6672706

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	3.376034	0.4822906	14.08	0.0002
REP	2	0.8730512	0.4365256	12.74	0.0018
LEVEL	2	1.5608017	0.7804009	2.48	0.2309
C(LEV)	3	0.9421811	0.3140604	9.17	0.0032
CULT	5	2.5029828	0.5005966	14.61	0.0003
Error	10	0.3426356	0.0342636		
Total	17	3.7186695			

R-Square	C.V.	R-MSE	Mean
0.907861	8.40908	0.1851	2.2012418

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	14.024455	2.0034936	1.26	0.364
REP	2	3.1246595	1.5623297	0.98	0.4106
LEVEL	2	5.7677691	2.8838845	1.89	0.2948
C(LEV)	3	4.5864246	1.5288082	0.96	0.4513
CULT	5	10.377679	2.0755358	1.31	0.3417
Error	9	14.286968	1.5874409		
Total	16	28.311423			

R-Square	C.V.	R-MSE	Mean
0.495364	72.19611	1.25994	1.7451588

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	33.266319	4.7523312	1.48	0.2762
REP	2	15.188991	7.5944957	2.37	0.1439
LEVEL	2	14.581263	7.2906317	6.26	0.085
C(LEV)	3	3.4960637	1.1653546	0.36	0.7809
CULT	5	18.077327	3.6154654	1.13	0.4059
Error	10	32.067375	3.2067375		
Total	17	65.333694			

R-Square	C.V.	R-MSE	Mean
0.509176	79.10138	1.79074	2.26385

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	9.5076194	1.3582313	1.08	0.4404
REP	2	1.5668707	0.7834353	0.62	0.5557
LEVEL	2	3.2021765	1.6010882	1.01	0.461
C(LEV)	3	4.7385722	1.5795241	1.26	0.3409
CULT	5	7.9407487	1.5881497	1.26	0.3509
Error	10	12.567573	1.2567573		
Total	17	22.075192			

R-Square	C.V.	R-MSE	Mean
0.430692	51.70175	1.12105	2.1683056

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	120.92112	17.274445	4.03	0.0234
REP	2	17.221991	8.6109955	2.01	0.1845
LEVEL	2	42.882595	21.441297	1.06	0.4491
C(LEV)	3	60.816532	20.272177	4.73	0.0264
CULT	5	103.69913	20.739825	4.84	0.0165
Error	10	42.8211	4.28211		
Total	17	163.74222			

R-Square	C.V.	R-MSE	Mean
0.738485	62.80179	2.06933	3.2950111

8.2.2 ANOVA for June 1999 samples group

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DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	28.036636	4.0052338	2.51	0.0995
REP	2	0.6624648	0.3312324	0.21	0.8163
LEVEL	2	6.4397193	3.2198596	0.49	0.6554
C(LEV)	3	19.794974	6.5983248	4.14	0.0424
CULT	5	27.405671	5.4811342	3.44	0.0517
Error	9	14.355114	1.5950127		
Total	16	42.39175			

R-Square	C.V.	R-MSE	Mean
0.66137	101.6209	1.26294	1.2427941

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	4.5782733	0.6540391	3.56	0.0346
REP	2	0.0840855	0.0420428	0.23	0.7997
LEVEL	2	2.4034805	1.2017402	1.72	0.3173
C(LEV)	3	2.0907073	0.6969024	3.79	0.0474
CULT	5	4.4941878	0.8988376	4.89	0.016
Error	10	1.8394865	0.1839487		
Total	17	6.4177598			

R-Square	C.V.	R-MSE	Mean
0.713376	49.26337	0.42889	0.8706111

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	59.339679	8.477097	2.26	0.1268
REP	2	8.6631102	4.3315551	1.15	0.358
LEVEL	2	12.729791	6.3648956	0.62	0.5959
C(LEV)	3	30.884586	10.294862	2.74	0.1052
CULT	5	49.16573	9.833146	2.62	0.0993
Error	9	33.785473	3.7539415		
Total	16	93.125152			

R-Square	C.V.	R-MSE	Mean
0.637204	117.1125	1.93751	1.6544

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	208.51953	29.788505	3.42	0.0389
REP	2	17.884723	8.9423616	1.03	0.3932
LEVEL	2	85.419408	42.709704	1.22	0.41
C(LEV)	3	105.2154	35.071801	4.03	0.0407
CULT	5	190.63481	38.126962	4.38	0.0226
Error	10	87.128243	8.7128243		
Total	17	295.64778			

R-Square	C.V.	R-MSE	Mean
0.705297	132.069	2.95175	2.2350056

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	37.259553	5.3227933	1.78	0.1971
REP	2	7.33375	3.666875	1.23	0.3342
LEVEL	2	10.606878	5.3034389	0.82	0.5187
C(LEV)	3	19.318925	6.4396418	2.15	0.1571
CULT	5	29.925803	5.9851607	2	0.1642
Error	10	29.925327	2.9925327		
Total	17	67.184881			

R-Square	C.V.	R-MSE	Mean
0.554582	123.4644	1.72989	1.4011278

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	12.296161	1.7565944	19.61	0.0001
REP	2	0.1497976	0.0748988	0.84	0.4616
LEVEL	2	5.772147	2.8860735	1.36	0.3802
C(LEV)	3	6.3742164	2.1247388	23.72	0.0001
CULT	5	12.146363	2.4292727	27.12	0.0001
Error	10	0.8958507	0.0895851		
Total	17	13.192012			

R-Square	C.V.	R-MSE	Mean
0.932091	30.42724	0.29931	0.9836833

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	9.4627931	1.3518276	1.66	0.2359
REP	2	0.3256592	0.1628296	0.2	0.8227
LEVEL	2	4.1491712	2.0745856	1.1	0.4381
C(LEV)	3	5.6556463	1.8852154	2.31	0.1449
CULT	5	9.4287608	1.8857522	2.31	0.13
Error	9	7.3469698	0.81633		
Total	16	16.809763			

R-Square	C.V.	R-MSE	Mean
0.562934	79.4804	0.90351	1.1367706

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	205.05092	29.292989	3.09	0.0519
REP	2	25.818156	12.909078	1.36	0.2994
LEVEL	2	88.126027	44.063014	1.45	0.3624
C(LEV)	3	91.106737	30.368912	3.21	0.0704
CULT	5	179.23276	35.846553	3.79	0.0348
Error	10	94.666607	9.4666607		
Total	17	299.71753			

R-Square	C.V.	R-MSE	Mean
0.684147	134.3937	3.07679	2.2893889

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	9.495259	1.3564656	5.73	0.0071
REP	2	0.5280443	0.2640222	1.12	0.3653
LEVEL	2	3.8485895	1.9242948	1.13	0.4313
C(LEV)	3	5.1186251	1.7062084	7.21	0.0073
CULT	5	8.9672147	1.7934429	7.58	0.0035
Error	10	2.3668121	0.2366812		
Total	17	11.862071			

R-Square	C.V.	R-MSE	Mean
0.800472	24.1954	0.4865	2.0107088

8.2.2 ANOVA for June 1999 samples group

SCOPOLETIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	219.70909	31.387012	3.11	0.0512
REP	2	28.453851	14.226926	1.41	0.2889
LEVEL	2	94.396574	47.198287	1.46	0.3604
C(LEV)	3	96.858661	32.28622	3.2	0.0709
CULT	5	191.25524	38.251047	3.79	0.0347
Error	10	100.93668	10.093668		
Total	17	320.64577			

R-Square	C.V.	R-MSE	Mean
0.685208	66.07153	3.17705	4.8085056

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	876.67936	125.23991	6.79	0.0038
REP	2	23.4676	11.7338	0.64	0.5494
LEVEL	2	176.04393	88.021963	0.39	0.7071
C(LEV)	3	677.16783	225.72261	12.24	0.0011
CULT	5	853.21176	170.64235	9.25	0.0016
Error	10	184.39017	18.439017		
Total	17	1061.0695			

R-Square	C.V.	R-MSE	Mean
0.826222	35.85215	4.29407	11.977156

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	2628.5118	375.50168	3.45	0.0378
REP	2	304.0669	152.03345	1.4	0.2917
LEVEL	2	528.23941	264.1197	0.44	0.6793
C(LEV)	3	1796.2055	598.73515	5.5	0.0171
CULT	5	2324.4449	464.88897	4.27	0.0244
Error	10	1088.2369	108.82369		
Total	17	3716.7487			

R-Square	C.V.	R-MSE	Mean
0.707207	42.8448	10.4319	24.348017

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	2136.3753	305.19648	2.62	0.0812
REP	2	243.32658	121.66329	1.05	0.3871
LEVEL	2	121.02639	60.513196	0.1	0.9057
C(LEV)	3	1772.0224	590.67412	5.07	0.0217
CULT	5	1893.0488	378.60975	3.25	0.0531
Error	10	1164.1393	116.41393		
Total	17	3300.5146			

R-Square	C.V.	R-MSE	Mean
0.647286	49.56071	10.7895	21.770328

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	2.9035197	0.4147885	9.78	0.0009
REP	2	0.0232268	0.0116134	0.27	0.766
LEVEL	2	0.5662429	0.2831214	0.37	0.7201
C(LEV)	3	2.31405	0.77135	18.18	0.0002
CULT	5	2.8802929	0.5760586	13.58	0.0003
Error	10	0.4242153	0.0424215		
Total	17	3.327735			

R-Square	C.V.	R-MSE	Mean
0.872521	4.266969	0.20596	4.8269601

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	1594.4659	227.78084	1.93	0.1662
REP	2	258.31654	129.15827	1.1	0.3711
LEVEL	2	238.56617	119.28308	0.33	0.7445
C(LEV)	3	1097.5832	365.86107	3.11	0.0758
CULT	5	1336.1494	267.22987	2.27	0.1267
Error	10	1178.2073	117.82073		
Total	17	2772.6732			

R-Square	C.V.	R-MSE	Mean
0.575064	48.85966	10.8545	22.215722

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	5666.1553	809.45075	3.15	0.0492
REP	2	133.29159	66.645797	0.26	0.7763
LEVEL	2	2187.9113	1093.9556	0.98	0.4701
C(LEV)	3	3344.9524	1114.9841	4.35	0.0333
CULT	5	5532.8637	1106.5727	4.31	0.0237
Error	10	2566.1214	256.61214		
Total	17	8232.2767			

R-Square	C.V.	R-MSE	Mean
0.688285	65.37459	16.0191	24.503583

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	2033.6814	290.52592	3.41	0.0393
REP	2	74.398085	37.199042	0.44	0.6581
LEVEL	2	620.68927	310.34464	0.7	0.5647
C(LEV)	3	1338.5941	446.19802	5.23	0.0198
CULT	5	1959.2833	391.85667	4.6	0.0194
Error	10	852.53823	85.253823		
Total	17	2886.2197			

R-Square	C.V.	R-MSE	Mean
0.704618	45.6783	9.2333	20.213756

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	1516.8897	216.69853	1.92	0.169
REP	2	319.68313	159.84157	1.41	0.2878
LEVEL	2	210.72756	105.36378	0.32	0.748
C(LEV)	3	986.479	328.82633	2.91	0.0873
CULT	5	1197.2066	239.44131	2.12	0.1462
Error	10	1130.0476	113.00476		
Total	17	2646.9373			

R-Square	C.V.	R-MSE	Mean
0.573074	66.06612	10.6304	16.0905

8.2.2 ANOVA for June 1999 samples group

SCOPOLIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	21.996304	3.1423292	1.71	0.2129
REP	2	10.713581	5.3567907	2.92	0.1006
LEVEL	2	0.8593864	0.4296932	0.12	0.888
C(LEV)	3	10.423336	3.4744455	1.89	0.195
CULT	5	11.282723	2.2565446	1.23	0.3645
Error	10	18.37317	1.837317		
Total	17	40.369474			

R-Square	C.V.	R-MSE	Mean
0.544875	80.87704	1.35548	1.6759722

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	2363.8917	337.69882	0.99	0.4901
REP	2	239.35792	119.67896	0.35	0.7129
LEVEL	2	1241.1676	620.58378	2.11	0.2681
C(LEV)	3	883.36623	294.45541	0.86	0.4923
CULT	5	2124.5338	424.90676	1.24	0.3587
Error	10	3417.8791	341.78791		
Total	17	5781.7709			

R-Square	C.V.	R-MSE	Mean
0.408853	68.35353	18.4875	27.046894

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	144.34018	20.620026	1.06	0.4521
REP	2	61.126984	30.563492	1.57	0.2556
LEVEL	2	67.274687	33.637343	6.33	0.0838
C(LEV)	3	15.938511	5.3128369	0.27	0.8438
CULT	5	83.213198	16.64264	0.85	0.5428
Error	10	194.87838	19.487838		
Total	17	339.21856			

R-Square	C.V.	R-MSE	Mean
0.425508	67.17479	4.4145	6.5716667

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	5642.769	806.10985	5.67	0.0074
REP	2	215.60652	107.80326	0.76	0.4936
LEVEL	2	3055.3888	1527.6944	1.93	0.2889
C(LEV)	3	2371.7737	790.59122	5.56	0.0166
CULT	5	5427.1625	1085.4325	7.64	0.0034
Error	10	1421.4302	142.14302		
Total	17	7064.1991			

R-Square	C.V.	R-MSE	Mean
0.798784	41.63327	11.9224	28.63665

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	591.67016	84.524308	1.2	0.3846
REP	2	75.274856	37.637428	0.53	0.6026
LEVEL	2	399.6679	199.83395	5.14	0.1075
C(LEV)	3	116.7274	38.909133	0.55	0.6588
CULT	5	516.3953	103.27906	1.46	0.2842
Error	10	706.07159	70.607159		
Total	17	1297.7417			

R-Square	C.V.	R-MSE	Mean
0.455923	63.14814	8.40281	13.3065

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	3872.8989	553.27127	0.61	0.7378
REP	2	996.74686	498.37343	0.55	0.5943
LEVEL	2	621.94187	310.97094	0.41	0.6939
C(LEV)	3	2254.2102	751.40339	0.83	0.5086
CULT	5	2876.152	575.23041	0.63	0.6797
Error	10	9087.1627	908.71627		
Total	17	12960.062			

R-Square	C.V.	R-MSE	Mean
0.298833	95.17836	30.1449	31.672033

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	5359.0861	765.58373	3.79	0.0284
REP	2	65.87688	32.93844	0.16	0.8517
LEVEL	2	3234.7817	1617.3909	2.36	0.2425
C(LEV)	3	2058.4275	686.14252	3.4	0.0617
CULT	5	5293.2093	1058.6419	5.24	0.0127
Error	10	2019.8209	201.98209		
Total	17	7378.907			

R-Square	C.V.	R-MSE	Mean
0.726271	49.29857	14.212	28.828506

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	923.01017	131.8586	0.73	0.6513
REP	2	102.12661	51.063306	0.28	0.7591
LEVEL	2	343.91588	171.95794	1.08	0.4429
C(LEV)	3	476.96768	158.98923	0.88	0.4828
CULT	5	820.88356	164.17671	0.91	0.5112
Error	10	1802.1776	180.21776		
Total	17	2725.1878			

R-Square	C.V.	R-MSE	Mean
0.338696	60.99304	13.4245	22.009922

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	2.4586314	0.3512331	3.15	0.0494
REP	2	0.0614198	0.0307099	0.28	0.765
LEVEL	2	1.7105992	0.8552996	3.74	0.1533
C(LEV)	3	0.6866124	0.2288708	2.05	0.1706
CULT	5	2.3972116	0.4794423	4.3	0.0239
Error	10	1.1157648	0.1115765		
Total	17	3.5743962			

R-Square	C.V.	R-MSE	Mean
0.687845	6.80787	0.33403	4.9065367

8.2.2 ANOVA for June 1999 samples group

PPD-MARKER

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	0	0	.	.
REP	2	0	0	.	.
LEVEL	2	0	0	.	.
C(LEV)	3	0	0	.	.
CULT	5	0	0	.	.
Error	10	0	0		
Total	17	0			

R-Square	C.V.	R-MSE	Mean
0	.	0	0

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	31.933745	4.5619635	1.16	0.4021
REP	2	3.3772189	1.6886095	0.43	0.6626
LEVEL	2	9.8398381	4.9199191	0.79	0.5306
C(LEV)	3	18.716688	6.2388959	1.59	0.2539
CULT	5	28.556526	5.7113052	1.45	0.2877
Error	10	39.359941	3.9359941		
Total	17	71.293685			

R-Squa	C.V.	R-MSE	Mean
0.45	184.3633	1.983934	1.0761

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	0.0706734	0.0100962	1	0.4834
REP	2	0.0201924	0.0100962	1	0.4019
LEVEL	2	0.0201924	0.0100962	1	0.4648
C(LEV)	3	0.0302886	0.0100962	1	0.4323
CULT	5	0.050481	0.0100962	1	0.4651
Error	10	0.1009621	0.0100962		
Total	17	0.1716355			

R-Square	C.V.	R-MSE	Mean
0.411765	424.2641	0.10048	0.0236833

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	12.802223	1.828889	2.25	0.1179
REP	2	0.5174645	0.2587322	0.32	0.7341
LEVEL	2	3.3179689	1.6589844	0.56	0.6236
C(LEV)	3	8.9667895	2.9889298	3.68	0.0508
CULT	5	12.284758	2.4569517	3.03	0.064
Error	10	8.1137184	0.8113718		
Total	17	20.915941			

R-Square	C.V.	R-MSE	Mean
0.61208	105.6585	0.90076	0.8525222

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	0.319067	0.045581	3.79	0.0283
REP	2	0.0072244	0.0036122	0.3	0.7468
LEVEL	2	0.1241856	0.0620928	0.99	0.4668
C(LEV)	3	0.187657	0.0625523	5.21	0.0201
CULT	5	0.3118426	0.0623685	5.19	0.0132
Error	10	0.1201272	0.0120127		
Total	17	0.4391941			

R-Square	C.V.	R-MSE	Mean
0.726483	89.69927	0.1096	0.1221889

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	10.446279	1.4923256	1.29	0.3454
REP	2	2.041841	1.0209205	0.88	0.4438
LEVEL	2	0.0252666	0.0126333	0	0.9955
C(LEV)	3	8.3791716	2.7930572	2.41	0.1274
CULT	5	8.4044383	1.6808877	1.45	0.2873
Error	10	11.574365	1.1574365		
Total	17	22.020644			

R-Square	C.V.	R-MSE	Mean
0.474386	91.04062	1.07584	1.1817167

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	23.127402	3.3039145	0.95	0.5109
REP	2	3.6868536	1.8434268	0.53	0.6038
LEVEL	2	4.5039829	2.2519914	0.45	0.6735
C(LEV)	3	14.936565	4.978855	1.43	0.2904
CULT	5	19.440548	3.8881096	1.12	0.4093
Error	10	34.721113	3.4721113		
Total	17	57.848514			

R-Square	C.V.	R-MSE	Mean
0.399792	216.3204	1.86336	0.8613889

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	13.042649	1.8632356	1.19	0.3858
REP	2	3.1115286	1.5557643	1	0.4028
LEVEL	2	3.6780391	1.8390196	0.88	0.4996
C(LEV)	3	6.2530817	2.0843606	1.34	0.3171
CULT	5	9.9311209	1.9862242	1.27	0.3474
Error	10	15.59899	1.559899		
Total	17	28.641639			

R-Square	C.V.	R-MSE	Mean
0.455374	109.3399	1.24896	1.1422722

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	3.7683316	0.5383331	2.09	0.1407
REP	2	0.1428122	0.0714061	0.28	0.7638
LEVEL	2	0.1508806	0.0754403	0.07	0.9382
C(LEV)	3	3.4746389	1.158213	4.49	0.0305
CULT	5	3.6255194	0.7251039	2.81	0.0772
Error	10	2.5793943	0.2579394		
Total	17	6.347726			

R-Square	C.V.	R-MSE	Mean
0.593651	32.9408	0.50788	1.541788

8.2.2 ANOVA for June 1999 samples group

PHENOLIC CONTENT

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	3159.2916	451.32738	6.04	0.0059
REP	2	237.28385	118.64192	1.59	0.2519
LEVEL	2	352.01796	176.00898	0.21	0.8249
C(LEV)	3	2569.9898	856.66328	11.46	0.0014
CULT	5	2922.0078	584.40156	7.82	0.0031
Error	10	747.33838	74.733838		
Total	17	3906.63			

R-Square	C.V.	R-MSE	Mean
0.8087	32.21078	8.64487	26.838444

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	2413.4071	344.77244	4.66	0.0146
REP	2	539.58071	269.79036	3.64	0.0648
LEVEL	2	118.33482	59.167409	0.1	0.9068
C(LEV)	3	1755.4916	585.16386	7.9	0.0054
CULT	5	1873.8264	374.76528	5.06	0.0143
Error	10	740.35512	74.035512		
Total	17	3153.7622			

R-Square	C.V.	R-MSE	Mean
0.765247	36.69186	8.60439	23.450406

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	2250.3474	321.4782	15.72	0.0001
REP	2	121.76549	60.882747	2.98	0.0968
LEVEL	2	175.01372	87.506861	0.13	0.8792
C(LEV)	3	1953.5682	651.18939	31.84	0.0001
CULT	5	2128.5819	425.71638	20.82	0.0001
Error	10	204.51878	20.451878		
Total	17	2454.8662			

R-Square	C.V.	R-MSE	Mean
0.916688	21.49344	4.52238	21.040722

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	2095.6071	299.37245	5.02	0.0113
REP	2	249.24927	124.62464	2.09	0.1744
LEVEL	2	332.92542	166.46271	0.33	0.7421
C(LEV)	3	1513.4324	504.47748	8.46	0.0043
CULT	5	1846.3579	369.27157	6.19	0.0072
Error	10	596.13074	59.613074		
Total	17	2691.7379			

R-Square	C.V.	R-MSE	Mean
0.778533	34.10022	7.72095	22.641939

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	3.1336726	0.4476675	133.71	0.0001
REP	2	0.0966069	0.0483035	14.43	0.0011
LEVEL	2	0.5740557	0.2870278	0.35	0.7303
C(LEV)	3	2.46301	0.8210034	245.23	0.0001
CULT	5	3.0370657	0.6074132	181.43	0.0001
Error	10	0.0334795	0.003348		
Total	17	3.1671521			

R-Square	C.V.	R-MSE	Mean
0.989429	1.161653	0.05786	4.9809596

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	2418.5168	345.5024	10.47	0.0007
REP	2	28.03722	14.01861	0.42	0.6652
LEVEL	2	436.16219	218.0811	0.33	0.7392
C(LEV)	3	1954.3174	651.43912	19.74	0.0002
CULT	5	2390.4795	478.09591	14.48	0.0003
Error	10	330.07568	33.007568		
Total	17	2748.5924			

R-Square	C.V.	R-MSE	Mean
0.879911	25.73469	5.74522	22.324817

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	1466.1747	209.45352	12.77	0.0003
REP	2	198.37961	99.189806	6.05	0.019
LEVEL	2	828.04249	414.02124	2.82	0.2043
C(LEV)	3	439.75257	146.58419	8.94	0.0035
CULT	5	1267.7951	253.55901	15.46	0.0002
Error	10	163.9932	16.39932		
Total	17	1630.1679			

R-Square	C.V.	R-MSE	Mean
0.899401	17.71075	4.04961	22.865256

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	2268.6101	324.08715	5.56	0.0079
REP	2	141.6653	70.832648	1.21	0.3371
LEVEL	2	816.69502	408.34751	0.93	0.4835
C(LEV)	3	1310.2498	436.74992	7.49	0.0065
CULT	5	2126.9448	425.38896	7.29	0.004
Error	10	583.21529	58.321529		
Total	17	2851.8254			

R-Square	C.V.	R-MSE	Mean
0.795494	33.87769	7.63685	22.542428

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	1580.1266	225.73238	6.53	0.0044
REP	2	10.041572	5.0207862	0.15	0.8667
LEVEL	2	695.04895	347.52448	1.19	0.4161
C(LEV)	3	875.03612	291.67871	8.43	0.0043
CULT	5	1570.0851	314.01701	9.08	0.0018
Error	10	345.91957	34.591957		
Total	17	1926.0462			

R-Square	C.V.	R-MSE	Mean
0.820399	28.67522	5.88149	20.510717

8.2.2 ANOVA for June 1999 samples group

TANNIN

DAY 0

Source	DF	SS	MS	F	Pr > F
Model	7	9004133.4	1286304.8	3.1	0.0518
REP	2	1086192.8	543096.38	1.31	0.3131
LEVEL	2	1643240.1	821620.07	0.39	0.7055
C(LEV)	3	6274700.5	2091566.8	5.03	0.0222
CULT	5	7917940.7	1583588.1	3.81	0.0342
Error	10	4154523.3	415452.33		
Total	17	13158657			

R-Square	C.V.	R-MSE	Mean
0.684275	57.24768	644.556	1125.9075

DAY 4

Source	DF	SS	MS	F	Pr > F
Model	7	10758600	1536942.9	2.24	0.12
REP	2	1090209.5	545104.74	0.79	0.4788
LEVEL	2	2741779.9	1370889.9	0.59	0.6064
C(LEV)	3	6926611.1	2308870.4	3.36	0.0633
CULT	5	9668391	1933678.2	2.81	0.0769
Error	10	6869755.8	686975.58		
Total	17	17628356			

R-Square	C.V.	R-MSE	Mean
0.610301	82.88386	828.84	1000.0015

DAY 1

Source	DF	SS	MS	F	Pr > F
Model	7	8703954	1243422	1.74	0.2048
REP	2	641369.17	320684.59	0.45	0.65
LEVEL	2	2609780.6	1304890.3	0.72	0.5562
C(LEV)	3	5452804.2	1817601.4	2.55	0.1145
CULT	5	8062584.8	1612517	2.26	0.1274
Error	10	7127794	712779.4		
Total	17	15831748			

R-Square	C.V.	R-MSE	Mean
0.549778	70.72284	844.263	1193.7623

DAY 5

Source	DF	SS	MS	F	Pr > F
Model	7	4312731.7	616104.53	7.8	0.0022
REP	2	46092.895	23046.448	0.29	0.7531
LEVEL	2	2201196.5	1100598.3	1.6	0.3368
C(LEV)	3	2065442.3	688480.76	8.72	0.0038
CULT	5	4266638.8	853327.76	10.8	0.0009
Error	10	789771.4	78977.14		
Total	17	5102503.1			

R-Square	C.V.	R-MSE	Mean
0.845219	28.13536	281.029	998.84533

DAY 2

Source	DF	SS	MS	F	Pr > F
Model	7	1562080.3	223154.33	2.54	0.0882
REP	2	381861.45	190930.73	2.17	0.1647
LEVEL	2	408919.83	204459.92	0.8	0.5283
C(LEV)	3	771298.99	257099.66	2.92	0.0864
CULT	5	1180218.8	236043.77	2.68	0.0863
Error	10	879200.38	87920.038		
Total	17	2441280.7			

R-Square	C.V.	R-MSE	Mean
0.639861	34.96005	296.513	848.1485

DAY 6

Source	DF	SS	MS	F	Pr > F
Model	7	1535664.4	219380.63	1.6	0.2415
REP	2	465740.97	232870.48	1.7	0.2319
LEVEL	2	735422.75	367711.37	3.3	0.1748
C(LEV)	3	334500.68	111500.23	0.81	0.5155
CULT	5	1069923.4	213984.69	1.56	0.2568
Error	10	1372043.3	137204.33		
Total	17	2907707.7			

R-Square	C.V.	R-MSE	Mean
0.528136	42.32946	370.411	875.0667

DAY 3

Source	DF	SS	MS	F	Pr > F
Model	7	2935322.5	419331.78	4.19	0.0206
REP	2	256170.24	128085.12	1.28	0.3196
LEVEL	2	730592.41	365296.21	0.56	0.6203
C(LEV)	3	1948559.8	649519.95	6.5	0.0103
CULT	5	2679152.3	535830.45	5.36	0.0118
Error	10	999615.81	99961.581		
Total	17	3934938.3			

R-Square	C.V.	R-MSE	Mean
0.745964	38.73699	316.167	816.18883

DAY 7

Source	DF	SS	MS	F	Pr > F
Model	7	7523002.1	1074714.6	9.47	0.001
REP	2	238834.73	119417.37	1.05	0.3847
LEVEL	2	5277541.6	2638770.8	3.95	0.1446
C(LEV)	3	2006625.8	668875.26	5.9	0.0139
CULT	5	7284167.4	1456833.5	12.84	0.0004
Error	10	1134333.4	113433.34		
Total	17	8657335.5			

R-Square	C.V.	R-MSE	Mean
0.868974	29.4814	336.799	1142.4108

Ln CURVE AREA

Source	DF	SS	MS	F	Pr > F
Model	7	2.4215351	0.3459336	35.09	0.0001
REP	2	0.038413	0.0192065	1.95	0.193
LEVEL	2	0.9495858	0.4747929	0.99	0.4665
C(LEV)	3	1.4335364	0.4778455	48.47	0.0001
CULT	5	2.3831222	0.4766244	48.34	0.0001
Error	10	0.0985905	0.0098591		
Total	17	2.5201256			

R-Square	C.V.	R-MSE	Mean
0.960879	1.134399	0.09929	8.7528945

8.2.3 ANOVA for December 1999 samples group

	POX						SCOP-POX					
DAY 0	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	4.79E-06	4.4E-07	3.18	0.0144	Model	11	0.0004861	4.419E-05	2.46	0.0435
	REP	2	0	0	0.01	0.9873	REP	2	3.571E-05	1.786E-05	0.99	0.3895
	LEVEL	2	8.3E-07	4.2E-07	0.74	0.5117	LEVEL	2	0.0000461	2.305E-05	0.4	0.6853
	C(LEV)	7	3.95E-06	5.6E-07	4.13	0.0072	C(LEV)	7	0.0004043	5.775E-05	3.22	0.0216
	CULT	9	4.78E-06	5.3E-07	3.89	0.0069	CULT	9	0.0004504	5.004E-05	2.79	0.0307
	Error	18	2.46E-06	1.4E-07			Error	18	0.0003233	1.796E-05		
	Total	29	7.25E-06				Total	29	0.0008094			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.660342	27.246	0.00037	0.0013573			0.600544	71.06199	0.00424	0.0059642
DAY 1	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	1.308E-05	1.19E-06	10.71	0.0001	Model	11	0.0015648	0.0001423	1.55	0.1973
	REP	2	4.1E-07	2.1E-07	1.85	0.1863	REP	2	0.0002203	0.0001101	1.2	0.3241
	LEVEL	2	2.1E-07	1.1E-07	0.06	0.9422	LEVEL	2	0.0002287	0.0001144	0.72	0.5207
	C(LEV)	7	1.246E-05	1.78E-06	16.03	0.0001	C(LEV)	7	0.0011158	0.0001594	1.74	0.163
	CULT	9	1.267E-05	1.41E-06	12.68	0.0001	CULT	9	0.0013445	0.0001494	1.63	0.1809
	Error	18	0.000002	1.1E-07			Error	18	0.0016514	9.174E-05		
	Total	29	1.508E-05				Total	29	0.0032161			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.867485	17.21762	0.00033	0.0019354			0.486538	118.5237	0.00958	0.0080812
DAY 2	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	2.273E-05	2.07E-06	2.42	0.0466	Model	11	0.0015385	0.0001399	1.44	0.238
	REP	2	8.52E-06	4.26E-06	4.99	0.0189	REP	2	0.000398	0.000199	2.05	0.1581
	LEVEL	2	1.66E-06	8.3E-07	0.46	0.6477	LEVEL	2	0.0002926	0.0001463	1.21	0.3543
	C(LEV)	7	1.255E-05	1.79E-06	2.1	0.0972	C(LEV)	7	0.0008479	0.0001211	1.25	0.3297
	CULT	9	1.421E-05	1.58E-06	1.85	0.1279	CULT	9	0.0011406	0.0001267	1.3	0.301
	Error	18	1.538E-05	8.5E-07			Error	18	0.0017495	0.0000972		
	Total	29	3.812E-05				Total	29	0.003288			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.596413	44.41324	0.00092	0.0020816			0.467917	81.59666	0.00986	0.0120823
DAY 3	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	0.0000266	2.42E-06	7.3	0.0001	Model	11	0.0015415	0.0001401	2.25	0.061
	REP	2	1.008E-05	5.04E-06	15.23	0.0001	REP	2	0.0003349	0.0001674	2.69	0.095
	LEVEL	2	9.6E-07	4.8E-07	0.22	0.811	LEVEL	2	0.0006201	0.0003101	3.7	0.0801
	C(LEV)	7	1.555E-05	2.22E-06	6.71	0.0005	C(LEV)	7	0.0005865	8.379E-05	1.35	0.2862
	CULT	9	1.651E-05	1.83E-06	5.54	0.001	CULT	9	0.0012066	0.0001341	2.15	0.0793
	Error	18	5.96E-06	3.3E-07			Error	18	0.0011203	6.224E-05		
	Total	29	3.256E-05				Total	29	0.0026618			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.816981	36.53607	0.00058	0.0015747			0.579113	47.13786	0.00789	0.0167364
DAY 4	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	1.738E-05	1.58E-06	6.77	0.0002	Model	11	0.0057046	0.0005186	10.56	0.0001
	REP	2	1.33E-06	6.6E-07	2.84	0.0846	REP	2	0.0007474	0.0003737	7.61	0.004
	LEVEL	2	1.6E-07	8E-08	0.03	0.9662	LEVEL	2	0.0007665	0.0003833	0.64	0.5555
	C(LEV)	7	1.589E-05	2.27E-06	9.73	0.0001	C(LEV)	7	0.0041907	0.0005987	12.19	0.0001
	CULT	9	1.605E-05	1.78E-06	7.64	0.0001	CULT	9	0.0049572	0.0005508	11.22	0.0001
	Error	18	0.0000042	2.3E-07			Error	18	0.000884	4.911E-05		
	Total	29	2.158E-05				Total	29	0.0065886			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.805312	34.05562	0.00048	0.0014186			0.865826	29.65451	0.00701	0.0236322
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	0.0002071	1.882E-05	7.87	0.0001	Model	11	0.0104681	0.0009517	2.22	0.0646
	REP	2	3.559E-05	1.779E-05	7.44	0.0044	REP	2	0.0039551	0.0019775	4.61	0.0242
	LEVEL	2	8.01E-06	4.01E-06	0.17	0.8458	LEVEL	2	0.0027203	0.0013601	2.51	0.1507
	C(LEV)	7	0.0001635	2.335E-05	9.76	0.0001	C(LEV)	7	0.0037928	0.0005418	1.26	0.3223
	CULT	9	0.0001715	1.905E-05	7.96	0.0001	CULT	9	0.006513	0.0007237	1.69	0.1651
	Error	18	4.308E-05	2.39E-06			Error	18	0.0077261	0.0004292		
	Total	29	0.0002502				Total	29	0.0181942			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.827788	22.25551	0.00155	0.0069512			0.575354	41.3538	0.02072	0.0500989

8.2.3 ANOVA for December 1999 samples group

PPO

	Source	DF	SS	MS	F	Pr > F
DAY 0	Model	11	9E-08	1E-08	1	0.4827
	REP	2	1E-08	0	0.37	0.6939
	LEVEL	2	4E-08	2E-08	2.41	0.1597
	C(LEV)	7	5E-08	1E-08	0.87	0.5501
	CULT	9	9E-08	1E-08	1.14	0.3869
	Error	18	1.5E-07	1E-08		
	Total	29	2.5E-07			
			R-Square	C.V.	R-MSE	Mean
			0.379226	110.6594	9.2E-05	8.348E-05
DAY 1	Source	DF	SS	MS	F	Pr > F
	Model	11	1.887E-05	1.72E-06	12.15	0.0001
	REP	2	4.6E-07	2.3E-07	1.65	0.2206
	LEVEL	2	4.71E-06	2.36E-06	1.2	0.3551
	C(LEV)	7	1.369E-05	1.96E-06	13.86	0.0001
	CULT	9	0.0000184	2.04E-06	14.49	0.0001
	Error	18	2.54E-06	1.4E-07		
	Total	29	2.141E-05			
			R-Square	C.V.	R-MSE	Mean
			0.881322	54.94098	0.00038	0.0006838
DAY 2	Source	DF	SS	MS	F	Pr > F
	Model	11	3.057E-05	2.78E-06	9.23	0.0001
	REP	2	1.71E-06	8.5E-07	2.84	0.0849
	LEVEL	2	9.62E-06	4.81E-06	1.75	0.2419
	C(LEV)	7	1.924E-05	2.75E-06	9.13	0.0001
	CULT	9	2.886E-05	3.21E-06	10.65	0.0001
	Error	18	5.42E-06	0.0000003		
	Total	29	3.599E-05			
			R-Square	C.V.	R-MSE	Mean
			0.849418	47.12583	0.00055	0.0011643
DAY 3	Source	DF	SS	MS	F	Pr > F
	Model	11	3.922E-05	3.57E-06	3.7	0.0069
	REP	2	2.72E-06	1.36E-06	1.41	0.2695
	LEVEL	2	0.0000135	6.75E-06	2.05	0.1988
	C(LEV)	7	0.000023	3.29E-06	3.41	0.0168
	CULT	9	0.0000365	4.06E-06	4.21	0.0046
	Error	18	1.733E-05	9.6E-07		
	Total	29	5.654E-05			
			R-Square	C.V.	R-MSE	Mean
			0.693562	67.78595	0.00098	0.0014474
DAY 4	Source	DF	SS	MS	F	Pr > F
	Model	11	3.429E-05	3.12E-06	8.48	0.0001
	REP	2	0.0000003	1.5E-07	0.41	0.6671
	LEVEL	2	9.59E-06	0.0000048	1.38	0.3133
	C(LEV)	7	0.0000244	3.49E-06	9.48	0.0001
	CULT	9	3.399E-05	3.78E-06	10.27	0.0001
	Error	18	6.62E-06	3.7E-07		
	Total	29	4.091E-05			
			R-Square	C.V.	R-MSE	Mean
			0.838217	38.76186	0.00061	0.0015644
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F
	Model	11	0.0003359	3.053E-05	11.1	0.0001
	REP	2	0.0000057	2.85E-06	1.04	0.3746
	LEVEL	2	0.0001064	5.319E-05	1.66	0.2563
	C(LEV)	7	0.0002238	3.197E-05	11.63	0.0001
	CULT	9	0.0003302	3.668E-05	13.34	0.0001
	Error	18	4.949E-05	2.75E-06		
	Total	29	0.0003854			
			R-Square	C.V.	R-MSE	Mean
			0.871569	40.39835	0.00166	0.0041045

CAT

	Source	DF	SS	MS	F	Pr > F
DAY 0	Model	11	0.0000009	8E-08	2.56	0.0373
	REP	2	0.0000001	5E-08	1.56	0.2371
	LEVEL	2	1E-08	1E-08	0.05	0.9486
	C(LEV)	7	7.9E-07	1.1E-07	3.52	0.0147
	CULT	9	0.0000008	9E-08	2.78	0.031
	Error	18	5.8E-07	3E-08		
	Total	29	1.48E-06			
			R-Square	C.V.	R-MSE	Mean
			0.609806	45.13761	0.00018	0.0003974
DAY 1	Source	DF	SS	MS	F	Pr > F
	Model	11	7.3E-07	7E-08	2.44	0.0449
	REP	2	1E-08	0	0.17	0.8422
	LEVEL	2	1.2E-07	6E-08	0.7	0.5299
	C(LEV)	7	0.0000006	9E-08	3.16	0.0232
	CULT	9	7.2E-07	8E-08	2.94	0.0245
	Error	18	4.9E-07	3E-08		
	Total	29	1.21E-06			
			R-Square	C.V.	R-MSE	Mean
			0.598642	35.78036	0.00016	0.0004599
DAY 2	Source	DF	SS	MS	F	Pr > F
	Model	11	8.2E-07	7E-08	3.57	0.0083
	REP	2	3E-08	2E-08	0.73	0.4936
	LEVEL	2	0.0000001	5E-08	0.49	0.632
	C(LEV)	7	6.9E-07	0.0000001	4.73	0.0037
	CULT	9	7.9E-07	9E-08	4.2	0.0047
	Error	18	3.8E-07	2E-08		
	Total	29	1.19E-06			
			R-Square	C.V.	R-MSE	Mean
			0.68553	24.54648	0.00014	0.0005882
DAY 3	Source	DF	SS	MS	F	Pr > F
	Model	11	9.9E-07	9E-08	2.01	0.0915
	REP	2	1.2E-07	6E-08	1.35	0.2835
	LEVEL	2	0	0	0.01	0.9934
	C(LEV)	7	8.6E-07	1.2E-07	2.76	0.039
	CULT	9	8.7E-07	0.0000001	2.15	0.0796
	Error	18	0.0000008	4E-08		
	Total	29	1.79E-06			
			R-Square	C.V.	R-MSE	Mean
			0.550796	38.96671	0.00021	0.0005424
DAY 4	Source	DF	SS	MS	F	Pr > F
	Model	11	1.85E-06	1.7E-07	4.27	0.0033
	REP	2	6E-08	3E-08	0.76	0.4821
	LEVEL	2	1.7E-07	8E-08	0.36	0.7082
	C(LEV)	7	1.62E-06	2.3E-07	5.88	0.0011
	CULT	9	1.79E-06	0.0000002	5.04	0.0017
	Error	18	7.1E-07	4E-08		
	Total	29	2.55E-06			
			R-Square	C.V.	R-MSE	Mean
			0.722743	36.94359	0.0002	0.0005369
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F
	Model	11	1.124E-05	1.02E-06	5.42	0.0008
	REP	2	4.8E-07	2.4E-07	1.27	0.3053
	LEVEL	2	1.1E-07	5E-08	0.03	0.9659
	C(LEV)	7	1.065E-05	1.52E-06	8.07	0.0002
	CULT	9	1.076E-05	0.0000012	6.34	0.0005
	Error	18	3.39E-06	1.9E-07		
	Total	29	1.463E-05			
			R-Square	C.V.	R-MSE	Mean
			0.76805	21.12921	0.00043	0.0020552

8.2.3 ANOVA for December 1999 samples group

	SCOPOLETIN						SCOPOLIN					
DAY 0	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	620.73898	56.430816	2.23	0.0635	Model	11	252.08295	22.916632	1.89	0.1113
	REP	2	330.00052	165.00026	6.51	0.0074	REP	2	40.194893	20.097447	1.66	0.2185
	LEVEL	2	109.41814	54.709069	2.11	0.1916	LEVEL	2	52.161912	26.080956	1.14	0.3719
	C(LEV)	7	181.32032	25.902903	1.02	0.449	C(LEV)	7	159.72614	22.81802	1.88	0.1325
	CULT	9	290.73846	32.304273	1.28	0.3146	CULT	9	211.88805	23.543117	1.94	0.1104
	Error	18	455.97092	25.331718			Error	18	218.29257	12.127365		
	Total	29	1076.7099				Total	29	470.37552			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.576515	67.36842	5.03306	7.4709525			0.535919	121.9007	3.48244	2.8567818
DAY 1	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	6281.1159	571.01054	3.72	0.0067	Model	11	4945.7755	449.61596	7.44	0.0001
	REP	2	138.04612	69.023061	0.45	0.6448	REP	2	41.407234	20.703617	0.34	0.7144
	LEVEL	2	3658.7606	1829.3803	5.15	0.0421	LEVEL	2	477.13255	238.56628	0.38	0.6989
	C(LEV)	7	2484.3093	354.90132	2.31	0.072	C(LEV)	7	4427.2357	632.46225	10.47	0.0001
	CULT	9	6143.0698	682.56331	4.45	0.0035	CULT	9	4904.3683	544.92981	9.02	0.0001
	Error	18	2763.3267	153.51815			Error	18	1087.3502	60.408347		
	Total	29	9044.4427				Total	29	6033.1258			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.694472	48.16646	12.3902	25.723801			0.81977	54.11531	7.77228	14.362443
DAY 2	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	5269.0127	479.00116	1.98	0.0961	Model	11	5678.4313	516.22103	3.36	0.0111
	REP	2	576.49171	288.24586	1.19	0.3271	REP	2	107.96705	53.983523	0.35	0.7085
	LEVEL	2	419.96745	209.98373	0.34	0.7203	LEVEL	2	126.4965	63.248251	0.08	0.9228
	C(LEV)	7	4272.5536	610.3648	2.52	0.0541	C(LEV)	7	5443.9678	777.70968	5.06	0.0026
	CULT	9	4692.521	521.39123	2.15	0.0795	CULT	9	5570.4643	618.94048	4.03	0.0058
	Error	18	4360.8245	242.26803			Error	18	2766.3809	153.68783		
	Total	29	9629.8372				Total	29	8444.8123			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.547155	45.20135	15.565	34.434725			0.672417	48.34754	12.3971	25.641614
DAY 3	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	6872.8567	624.80515	2.77	0.0268	Model	11	8285.4834	753.22576	2.6	0.0348
	REP	2	79.699296	39.849648	0.18	0.8395	REP	2	89.722733	44.861367	0.15	0.8576
	LEVEL	2	508.1111	254.05555	0.28	0.7618	LEVEL	2	4637.8353	2318.9177	4.56	0.0539
	C(LEV)	7	6285.0463	897.86375	3.98	0.0085	C(LEV)	7	3557.9253	508.27505	1.76	0.1588
	CULT	9	6793.1574	754.79526	3.35	0.014	CULT	9	8195.7607	910.64008	3.15	0.0184
	Error	18	4061.1232	225.61796			Error	18	5211.8757	289.54865		
	Total	29	10933.98				Total	29	13497.359			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.628578	39.77268	15.0206	37.766085			0.61386	43.30715	17.0161	39.29173
DAY 4	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	11507.851	1046.1683	4.28	0.0032	Model	11	15782.849	1434.8045	5.52	0.0007
	REP	2	222.38662	111.19331	0.46	0.6413	REP	2	1025.2621	512.63106	1.97	0.1682
	LEVEL	2	278.8079	139.40395	0.09	0.9162	LEVEL	2	3125.9454	1562.9727	0.94	0.4347
	C(LEV)	7	11006.657	1572.3795	6.44	0.0007	C(LEV)	7	11631.642	1661.6631	6.39	0.0007
	CULT	9	11285.465	1253.9405	5.13	0.0016	CULT	9	14757.587	1639.7319	6.31	0.0005
	Error	18	4395.6474	244.20263			Error	18	4680.8043	260.04468		
	Total	29	15903.499				Total	29	20463.653			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.723605	47.05838	15.627	33.207652			0.771263	41.65556	16.1259	38.71248
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F	Source	DF	SS	MS	F	Pr > F
	Model	11	3.4549489	0.3140863	3.07	0.0169	Model	11	6.0725993	0.5520545	5.81	0.0005
	REP	2	0.0253273	0.0126636	0.12	0.8842	REP	2	0.1835234	0.0917617	0.97	0.3998
	LEVEL	2	0.7857221	0.392861	1.04	0.4022	LEVEL	2	0.9852938	0.4926469	0.7	0.5269
	C(LEV)	7	2.6438995	0.3776999	3.7	0.0119	C(LEV)	7	4.9037822	0.7005403	7.37	0.0003
	CULT	9	3.4296216	0.3810691	3.73	0.0084	CULT	9	5.889076	0.6543418	6.88	0.0003
	Error	18	1.8389277	0.1021627			Error	18	1.7114288	0.0950794		
	Total	29	5.2938766				Total	29	7.7840281			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.652631	6.804912	0.31963	4.6970327			0.780136	6.868534	0.30835	4.4893046

8.2.3 ANOVA for December 1999 samples group

ESCULIN

	Source	DF	SS	MS	F	Pr > F
DAY 0	Model	11	0.0766703	0.00697	3.42	0.0103
	REP	2	0.0040806	0.0020403	1	0.3874
	LEVEL	2	0.0120983	0.0060491	0.7	0.5283
	C(LEV)	7	0.0604914	0.0086416	4.24	0.0063
	CULT	9	0.0725897	0.0080655	3.95	0.0063
	Error	18	0.0367251	0.0020403		
	Total	29	0.1133954			
			R-Square	C.V.	R-MSE	Mean
			0.676132	275.4796	0.04517	0.0163967
DAY 1	Source	DF	SS	MS	F	Pr > F
	Model	11	3.6062548	0.3278414	1.45	0.2332
	REP	2	0.5019994	0.2509997	1.11	0.3508
	LEVEL	2	0.6590486	0.3295243	0.94	0.4338
	C(LEV)	7	2.4452069	0.3493153	1.55	0.2148
	CULT	9	3.1042555	0.3449173	1.53	0.2124
	Error	18	4.0665026	0.2259168		
	Total	29	7.6727574			
			R-Square	C.V.	R-MSE	Mean
			0.470008	166.2642	0.47531	0.2858746
DAY 2	Source	DF	SS	MS	F	Pr > F
	Model	11	82.277133	7.4797394	3.04	0.0176
	REP	2	5.9761441	2.9880721	1.22	0.3196
	LEVEL	2	30.945188	15.472594	2.39	0.1619
	C(LEV)	7	45.355801	6.4794001	2.64	0.0461
	CULT	9	76.300989	8.4778877	3.45	0.0122
	Error	18	44.226535	2.4570297		
	Total	29	126.50367			
			R-Square	C.V.	R-MSE	Mean
			0.650393	116.4612	1.56749	1.3459349
DAY 3	Source	DF	SS	MS	F	Pr > F
	Model	11	330.57886	30.052623	2.12	0.0752
	REP	2	9.659538	4.829769	0.34	0.7152
	LEVEL	2	147.14952	73.574761	2.96	0.1168
	C(LEV)	7	173.7698	24.824257	1.76	0.1589
	CULT	9	320.91932	35.657702	2.52	0.0454
	Error	18	254.57385	14.142992		
	Total	29	585.1527			
			R-Square	C.V.	R-MSE	Mean
			0.564945	128.0326	3.76072	2.9373122
DAY 4	Source	DF	SS	MS	F	Pr > F
	Model	11	207.90386	18.900351	1.39	0.2573
	REP	2	1.7991938	0.8995969	0.07	0.9361
	LEVEL	2	26.284921	13.14246	0.51	0.6203
	C(LEV)	7	179.81974	25.688535	1.89	0.1304
	CULT	9	206.10467	22.900518	1.69	0.1648
	Error	18	244.30499	13.5725		
	Total	29	452.20885			
			R-Square	C.V.	R-MSE	Mean
			0.459752	137.2894	3.68409	2.6834457
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F
	Model	11	11.867199	1.0788362	1.93	0.1047
	REP	2	0.0749585	0.0374792	0.07	0.9355
	LEVEL	2	6.4456038	3.2228019	4.22	0.0628
	C(LEV)	7	5.3466364	0.7638052	1.36	0.2791
	CULT	9	11.79224	1.3102489	2.34	0.0597
	Error	18	10.081977	0.5601099		
	Total	29	21.949176			
			R-Square	C.V.	R-MSE	Mean
			0.540667	48.81968	0.7484	1.5329984

PPD-MARKER

	Source	DF	SS	MS	F	Pr > F
DAY 0	Model	11	0	0	.	.
	REP	2	0	0	.	.
	LEVEL	2	0	0	.	.
	C(LEV)	7	0	0	.	.
	CULT	9	0	0	.	.
	Error	18	0	0		
	Total	29	0			
			R-Square	C.V.	R-MSE	Mean
			0	.	0	0
DAY 1	Source	DF	SS	MS	F	Pr > F
	Model	11	0.0994807	0.0090437	1.82	0.1245
	REP	2	0.0063922	0.0031961	0.64	0.5367
	LEVEL	2	0.0423677	0.0211839	2.92	0.1194
	C(LEV)	7	0.0507208	0.0072458	1.46	0.2429
	CULT	9	0.0930885	0.0103432	2.08	0.0882
	Error	18	0.089297	0.0049609		
	Total	29	0.1887777			
			R-Square	C.V.	R-MSE	Mean
			0.526973	127.6899	0.07043	0.0551602
DAY 2	Source	DF	SS	MS	F	Pr > F
	Model	11	7.736805	0.7033459	2.6	0.0348
	REP	2	1.5754511	0.7877256	2.91	0.0801
	LEVEL	2	3.2638942	1.6319471	3.94	0.0713
	C(LEV)	7	2.8974596	0.4139228	1.53	0.2195
	CULT	9	6.1613539	0.6845949	2.53	0.0446
	Error	18	4.8662522	0.2703474		
	Total	29	12.603057			
			R-Square	C.V.	R-MSE	Mean
			0.613883	108.346	0.51995	0.4798972
DAY 3	Source	DF	SS	MS	F	Pr > F
	Model	11	26.367927	2.3970843	2.36	0.0508
	REP	2	4.5787614	2.2893807	2.26	0.1333
	LEVEL	2	15.260961	7.6304805	8.18	0.0147
	C(LEV)	7	6.5282047	0.9326007	0.92	0.514
	CULT	9	21.789166	2.4210184	2.39	0.0554
	Error	18	18.245285	1.0136269		
	Total	29	44.613212			
			R-Square	C.V.	R-MSE	Mean
			0.591034	78.66836	1.00679	1.2797907
DAY 4	Source	DF	SS	MS	F	Pr > F
	Model	11	40.138428	3.648948	1.69	0.1562
	REP	2	6.6718621	3.3359311	1.54	0.2405
	LEVEL	2	6.8701547	3.4350774	0.9	0.4474
	C(LEV)	7	26.596411	3.7994873	1.76	0.1581
	CULT	9	33.466566	3.7185073	1.72	0.1562
	Error	18	38.891966	2.1606648		
	Total	29	79.030394			
			R-Square	C.V.	R-MSE	Mean
			0.507886	84.35722	1.46992	1.7424947
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F
	Model	11	7.9105606	0.7191419	4.04	0.0044
	REP	2	2.1588921	1.079446	6.07	0.0097
	LEVEL	2	3.655682	1.827841	6.1	0.0292
	C(LEV)	7	2.0959866	0.2994267	1.68	0.1762
	CULT	9	5.7516685	0.6390743	3.59	0.0101
	Error	18	3.2019339	0.1778852		
	Total	29	11.112494			
			R-Square	C.V.	R-MSE	Mean
			0.711862	37.61135	0.42176	1.1213754

8.2.3 ANOVA for December 1999 samples group

	Source	DF	SS	MS	F	Pr > F
DAY 0	Model	11	4845.1465	440.46786	4.48	0.0025
	REP	2	876.98104	438.49052	4.46	0.0268
	LEVEL	2	4.3408318	2.1704159	0	0.9962
	C(LEV)	7	3963.8246	566.26066	5.76	0.0013
	CULT	9	3968.1654	440.90727	4.48	0.0033
	Error	18	1770.5674	98.364857		
	Total	29	6615.7139			
			R-Square	C.V.	R-MSE	Mean
			0.732369	34.9752	9.91791	28.356967
DAY 1	Source	DF	SS	MS	F	Pr > F
	Model	11	3180.4924	289.13567	2.94	0.0207
	REP	2	312.85404	156.42702	1.59	0.2314
	LEVEL	2	493.3654	246.6827	0.73	0.5164
	C(LEV)	7	2374.2729	339.18185	3.45	0.0161
	CULT	9	2867.6383	318.62648	3.24	0.0162
	Error	18	1771.4019	98.411215		
	Total	29	4951.8942			
			R-Square	C.V.	R-MSE	Mean
			0.642278	35.31424	9.92024	28.091338
DAY 2	Source	DF	SS	MS	F	Pr > F
	Model	11	2491.8875	226.53523	4.12	0.0039
	REP	2	171.16843	85.584217	1.56	0.238
	LEVEL	2	681.36647	340.68323	1.45	0.2963
	C(LEV)	7	1639.3526	234.19323	4.26	0.0062
	CULT	9	2320.7191	257.85768	4.69	0.0026
	Error	18	989.88111	54.993395		
	Total	29	3481.7686			
			R-Square	C.V.	R-MSE	Mean
			0.715696	25.85726	7.41575	28.679582
DAY 3	Source	DF	SS	MS	F	Pr > F
	Model	11	1405.9929	127.81754	1.67	0.1605
	REP	2	331.14202	165.57101	2.17	0.1435
	LEVEL	2	90.192753	45.096376	0.32	0.7358
	C(LEV)	7	984.65816	140.66545	1.84	0.1405
	CULT	9	1074.8509	119.42788	1.56	0.2006
	Error	18	1375.5308	76.418376		
	Total	29	2781.5237			
			R-Square	C.V.	R-MSE	Mean
			0.505476	31.1584	8.74176	28.055875
DAY 4	Source	DF	SS	MS	F	Pr > F
	Model	11	2382.8947	216.62679	5.28	0.001
	REP	2	151.30029	75.650147	1.84	0.1869
	LEVEL	2	384.60822	192.30411	0.73	0.5158
	C(LEV)	7	1846.9862	263.85517	6.43	0.0007
	CULT	9	2231.5944	247.95494	6.04	0.0006
	Error	18	738.63192	41.035107		
	Total	29	3121.5266			
			R-Square	C.V.	R-MSE	Mean
			0.763375	20.69164	6.40587	30.958717
Ln CURVE AREA	Source	DF	SS	MS	F	Pr > F
	Model	11	2.130403	0.193673	6.77	0.0002
	REP	2	0.3191429	0.1595715	5.58	0.013
	LEVEL	2	0.227644	0.113822	0.5	0.6249
	C(LEV)	7	1.5836162	0.2262309	7.91	0.0002
	CULT	9	1.8112601	0.2012511	7.03	0.0002
	Error	18	0.5149331	0.0286074		
	Total	29	2.6453362			
			R-Square	C.V.	R-MSE	Mean
			0.805343	3.594446	0.16914	4.7055152

8.2.4 ANOVA for Family K samples group

	POX						SCP-POX					
DAY 0	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	5.59E-06	1.6E-07	1.5	0.0748	Model	36	0.0006385	1.774E-05	1.35	0.143
	REP	2	2.7E-07	1.4E-07	1.31	0.2764	REP	2	3.276E-05	1.638E-05	1.25	0.2941
	LEVEL	2	1.08E-06	5.4E-07	4.09	0.0261	LEVEL	2	1.352E-05	6.76E-06	0.37	0.6969
	CULT	34	5.32E-06	1.6E-07	1.51	0.0741	CULT	34	0.0006058	1.782E-05	1.36	0.1429
	Error	68	7.03E-06	0.0000001			Error	68	0.0008938	1.314E-05		
	Total	104	1.262E-05				Total	104	0.0015323			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.442779	24.36078	0.00032	0.00132			0.416703	83.19428	0.00363	0.0043578
DAY 1	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	6.966E-05	1.93E-06	2.71	0.0002	Model	36	0.0002343	6.51E-06	1.74	0.0254
	REP	2	5.88E-06	2.94E-06	4.11	0.0206	REP	2	1.265E-05	6.32E-06	1.69	0.1929
	LEVEL	2	9.41E-06	4.71E-06	2.77	0.0777	LEVEL	2	8.22E-06	4.11E-06	0.62	0.5464
	CULT	34	6.378E-05	1.88E-06	2.63	0.0004	CULT	34	0.0002217	6.52E-06	1.74	0.0267
	Error	68	4.859E-05	7.1E-07			Error	68	0.0002551	3.75E-06		
	Total	104	0.0001183				Total	104	0.0004894			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.589075	44.1594	0.00085	0.0019143			0.478769	35.03645	0.00194	0.005528
DAY 2	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	41.343002	1.1484167	1	0.4875	Model	36	0.003371	9.364E-05	1.34	0.1484
	REP	2	2.3049153	1.1524577	1	0.3718	REP	2	0.0012964	0.0006482	9.28	0.0003
	LEVEL	2	1.5326774	0.7663387	0.65	0.5268	LEVEL	2	3.577E-05	1.789E-05	0.28	0.7571
	CULT	34	39.038086	1.148179	1	0.4866	CULT	34	0.0020746	6.102E-05	0.87	0.6616
	Error	68	78.062716	1.1479811			Error	68	0.0047508	6.987E-05		
	Total	104	119.40572				Total	104	0.0081218			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.34624	1007.18	1.07144	0.1063801			0.415051	70.69023	0.00836	0.0118242
DAY 3	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	4.214E-05	1.17E-06	3	0.0001	Model	36	0.0062272	0.000173	2.19	0.0026
	REP	2	1.303E-05	6.51E-06	16.69	0.0001	REP	2	0.0009408	0.0004704	5.96	0.0041
	LEVEL	2	8.26E-06	4.13E-06	6.34	0.0048	LEVEL	2	0.001708	0.000854	7.64	0.0019
	CULT	34	2.912E-05	8.6E-07	2.19	0.003	CULT	34	0.0052865	0.0001555	1.97	0.0088
	Error	68	2.654E-05	3.9E-07			Error	68	0.0053627	7.886E-05		
	Total	104	6.868E-05				Total	104	0.0115899			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.61359	32.88107	0.00062	0.0019			0.537297	40.62281	0.00888	0.0218609
DAY 4	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	4.128E-05	1.15E-06	2.94	0.0001	Model	36	0.0168612	0.0004684	2.54	0.0005
	REP	2	1.072E-05	5.36E-06	13.75	0.0001	REP	2	0.0024594	0.0012297	6.66	0.0023
	LEVEL	2	6.05E-06	3.02E-06	3.95	0.0294	LEVEL	2	0.0013056	0.0006528	1.6	0.2186
	CULT	34	3.057E-05	0.0000009	2.31	0.0017	CULT	34	0.0144018	0.0004236	2.3	0.0018
	Error	68	0.0000265	3.9E-07			Error	68	0.0125463	0.0001845		
	Total	104	6.779E-05				Total	104	0.0294075			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.609028	35.15549	0.00062	0.0017758			0.573363	40.26862	0.01358	0.0337316
Ln CURVE AREA	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	2.1116281	0.0586563	1.01	0.48	Model	36	0.0264179	0.0007338	2.43	0.0008
	REP	2	0.1219569	0.0609785	1.05	0.357	REP	2	0.0071356	0.0035678	11.8	0.0001
	LEVEL	2	0.081684	0.040842	0.68	0.5113	LEVEL	2	0.0028182	0.0014091	2.74	0.0798
	CULT	34	1.9896712	0.0585197	1	0.482	CULT	34	0.0192824	0.0005671	1.87	0.014
	Error	68	3.9650989	0.0583103			Error	68	0.020568	0.0003025		
	Total	104	6.0767271				Total	104	0.0469859			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.347494	786.0288	0.24148	0.0307209			0.562252	30.83678	0.01739	0.0563991

8.2.4 ANOVA for Family K samples group

	PPO						SCOPOLETIN					
DAY 0	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	8.8E-07	2E-08	1.44	0.0959	Model	36	2189.7796	60.827212	2.8	0.0001
	REP	2	1E-08	0	0.17	0.8481	REP	2	698.43749	349.21874	16.08	0.0001
	LEVEL	2	2E-08	1E-08	0.32	0.725	LEVEL	2	195.40687	97.703437	2.41	0.1057
	CULT	34	8.7E-07	3E-08	1.52	0.0718	CULT	34	1491.3422	43.863005	2.02	0.007
	Error	68	1.14E-06	2E-08			Error	68	1476.5124	21.713417		
	Total	104	2.02E-06				Total	104	3666.292			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.433307	136.3573	0.00013	9.514E-05			0.597274	61.66832	4.65977	7.5561749
DAY 1	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	2.452E-05	6.8E-07	3.14	0.0001	Model	36	4268.8777	118.57994	1.89	0.0121
	REP	2	2.58E-06	1.29E-06	5.94	0.0042	REP	2	109.61123	54.805615	0.87	0.4225
	LEVEL	2	5.4E-07	2.7E-07	0.4	0.6705	LEVEL	2	738.14958	369.07479	3.45	0.0439
	CULT	34	2.194E-05	6.5E-07	2.97	0.0001	CULT	34	4159.2665	122.33137	1.95	0.0099
	Error	68	1.477E-05	2.2E-07			Error	68	4270.4828	62.801218		
	Total	104	3.929E-05				Total	104	8539.3605			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.624118	71.29149	0.00047	0.0006537			0.499906	41.62043	7.92472	19.040463
DAY 2	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	6.785E-05	1.88E-06	3.38	0.0001	Model	36	29766.354	826.84317	2.48	0.0006
	REP	2	1.77E-06	8.8E-07	1.58	0.2129	REP	2	2357.7649	1178.8824	3.54	0.0344
	LEVEL	2	3.62E-06	1.81E-06	0.93	0.4056	LEVEL	2	13276	6638.0001	15.03	0.0001
	CULT	34	6.608E-05	1.94E-06	3.49	0.0001	CULT	34	27408.589	806.13498	2.42	0.001
	Error	68	3.792E-05	5.6E-07			Error	68	22635.677	332.8776		
	Total	104	0.0001058				Total	104	52402.031			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.641463	61.99979	0.00075	0.0012045			0.568038	46.33344	18.2449	39.377464
DAY 3	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	6.273E-05	1.74E-06	4.5	0.0001	Model	36	28601.432	794.48423	2.82	0.0001
	REP	2	8.84E-06	4.42E-06	11.42	0.0001	REP	2	2208.9902	1104.4951	3.92	0.0245
	LEVEL	2	2.24E-06	1.12E-06	0.69	0.5069	LEVEL	2	9777.4307	4888.7153	9.42	0.0006
	CULT	34	0.0000539	1.59E-06	4.1	0.0001	CULT	34	26392.442	776.24829	2.75	0.0002
	Error	68	2.631E-05	3.9E-07			Error	68	19159.742	281.76092		
	Total	104	8.904E-05				Total	104	47761.175			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.704519	45.79865	0.00062	0.0013582			0.598843	41.90714	16.7857	40.0546
DAY 4	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	0.000064	1.78E-06	3.04	0.0001	Model	36	27376.907	760.46963	2.46	0.0007
	REP	2	1.5E-07	7E-08	0.12	0.8827	REP	2	3088.6598	1544.3299	4.99	0.0095
	LEVEL	2	6.42E-06	3.21E-06	1.79	0.1833	LEVEL	2	4256.8182	2128.4091	3.4	0.0458
	CULT	34	6.385E-05	1.88E-06	3.21	0.0001	CULT	34	24288.247	714.36021	2.31	0.0017
	Error	68	3.976E-05	5.8E-07			Error	68	21028.442	309.2418		
	Total	104	0.0001038				Total	104	48405.349			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.616814	50.38415	0.00076	0.0015176			0.565576	51.21231	17.5853	34.337977
Ln CURVE AREA	Source	DF	SS	MS	F Value	Pr > F	Source	DF	SS	MS	F Value	Pr > F
	Model	36	0.0005227	1.452E-05	6.59	0.0001	Model	36	13.849241	0.3847011	4.42	0.0001
	REP	2	2.849E-05	1.424E-05	6.46	0.0027	REP	2	0.403873	0.2019365	2.32	0.106
	LEVEL	2	2.827E-05	1.414E-05	0.97	0.3896	LEVEL	2	6.1087187	3.0543593	13.32	0.0001
	CULT	34	0.0004943	1.454E-05	6.6	0.0001	CULT	34	13.445368	0.395452	4.54	0.0001
	Error	68	0.0001498	0.0000022			Error	68	5.9191927	0.087047		
	Total	104	0.0006726				Total	104	19.768433			
			R-Square	C.V.	R-MSE	Mean			R-Square	C.V.	R-MSE	Mean
			0.777241	37.00157	0.00148	0.0040115			0.700574	6.276468	0.29504	4.7006882

8.2.4 ANOVA for Family K samples group

SCOPOLIN

	Source	DF	SS	MS	F Value	Pr > F
DAY 0	Model	36	1431.9128	39.775356	1.07	0.3933
	REP	2	38.158196	19.079098	0.51	0.6
	LEVEL	2	19.577516	9.7887582	0.23	0.7974
	CULT	34	1393.7546	40.992783	1.11	0.3552
	Error	68	2521.146	37.075676		
	Total	104	3953.0588			
			R-Square	C.V.	R-MSE	Mean
			0.362229	158.4797	6.08898	3.8421198
DAY 1	Source	DF	SS	MS	F Value	Pr > F
	Model	36	5488.1935	152.44982	2.16	0.0032
	REP	2	1164.746	582.37299	8.24	0.0006
	LEVEL	2	136.5687	68.284351	0.52	0.5984
	CULT	34	4323.4475	127.16022	1.8	0.02
	Error	68	4803.9075	70.645698		
	Total	104	10292.101			
			R-Square	C.V.	R-MSE	Mean
			0.533243	60.70962	8.4051	13.844758
DAY 2	Source	DF	SS	MS	F Value	Pr > F
	Model	36	18642.602	517.85005	2.68	0.0002
	REP	2	5050.8658	2525.4329	13.06	0.0001
	LEVEL	2	912.55914	456.27957	1.15	0.3289
	CULT	34	13591.736	399.75694	2.07	0.0055
	Error	68	13146.442	193.33003		
	Total	104	31789.044			
			R-Square	C.V.	R-MSE	Mean
			0.586447	41.79954	13.9043	33.264281
DAY 3	Source	DF	SS	MS	F Value	Pr > F
	Model	36	28822.388	800.62189	3.22	0.0001
	REP	2	3155.13	1577.565	6.35	0.003
	LEVEL	2	5162.9953	2581.4976	4.03	0.0275
	CULT	34	25667.258	754.91935	3.04	0.0001
	Error	68	16888.681	248.36295		
	Total	104	45711.069			
			R-Square	C.V.	R-MSE	Mean
			0.630534	36.12524	15.7595	43.624726
DAY 4	Source	DF	SS	MS	F Value	Pr > F
	Model	36	27173.278	754.81329	1.52	0.0702
	REP	2	1199.8028	599.90138	1.2	0.3062
	LEVEL	2	5200.2395	2600.1197	4.01	0.028
	CULT	34	25973.476	763.92576	1.53	0.0675
	Error	68	33874.65	498.15661		
	Total	104	61047.928			
			R-Square	C.V.	R-MSE	Mean
			0.445114	45.3804	22.3194	49.182955
Ln CURVE AREA	Source	DF	SS	MS	F Value	Pr > F
	Model	36	12.718249	0.3532847	5.24	0.0001
	REP	2	1.8989277	0.9494639	14.1	0.0001
	LEVEL	2	1.5998129	0.7999064	2.78	0.0773
	CULT	34	10.819321	0.3182153	4.72	0.0001
	Error	68	4.5805899	0.0673616		
	Total	104	17.298838			
			R-Square	C.V.	R-MSE	Mean
			0.735208	5.526208	0.25954	4.6965509

ESCULIN

	Source	DF	SS	MS	F Value	Pr > F
DAY 0	Model	36	3.4835452	0.0967652	3.64	0.0001
	REP	2	0.0173741	0.008687	0.33	0.7221
	LEVEL	2	0.1001599	0.05008	0.48	0.6255
	CULT	34	3.4661712	0.1019462	3.84	0.0001
	Error	68	1.805883	0.0265571		
	Total	104	5.2894282			
			R-Square	C.V.	R-MSE	Mean
			0.658586	175.4182	0.16296	0.0929
DAY 1	Source	DF	SS	MS	F Value	Pr > F
	Model	36	20.046607	0.5568502	1.05	0.4271
	REP	2	0.0128182	0.0064091	0.01	0.988
	LEVEL	2	1.1514183	0.5757091	0.98	0.3879
	CULT	34	20.033789	0.5892291	1.11	0.3539
	Error	68	36.200521	0.5323606		
	Total	104	56.247128			
			R-Square	C.V.	R-MSE	Mean
			0.356402	154.4661	0.72963	0.4723564
DAY 2	Source	DF	SS	MS	F Value	Pr > F
	Model	36	100.02437	2.7784548	1.68	0.0331
	REP	2	22.431495	11.215747	6.79	0.0021
	LEVEL	2	19.209513	9.6047564	5.28	0.0104
	CULT	34	78.51479	2.3092585	1.4	0.1211
	Error	67	110.69782	1.6522063		
	Total	103	210.72219			
			R-Square	C.V.	R-MSE	Mean
			0.474674	85.22519	1.28538	1.5082181
DAY 3	Source	DF	SS	MS	F Value	Pr > F
	Model	36	196.01935	5.4449819	2.29	0.0017
	REP	2	17.718401	8.8592003	3.72	0.0293
	LEVEL	2	46.433785	23.216892	5.63	0.008
	CULT	34	178.30095	5.2441456	2.2	0.0029
	Error	68	161.95841	2.3817413		
	Total	104	357.97776			
			R-Square	C.V.	R-MSE	Mean
			0.547574	73.01319	1.54329	2.1137128
DAY 4	Source	DF	SS	MS	F Value	Pr > F
	Model	36	256.30953	7.1197091	2.85	0.0001
	REP	2	24.515388	12.257694	4.91	0.0102
	LEVEL	2	65.2896	32.6448	6.3	0.0049
	CULT	34	230.07828	6.7670083	2.71	0.0002
	Error	67	167.11144	2.4942006		
	Total	103	423.42096			
			R-Square	C.V.	R-MSE	Mean
			0.60533	66.22059	1.5793	2.3849134
Ln CURVE AREA	Source	DF	SS	MS	F Value	Pr > F
	Model	36	23.038873	0.6399687	5.06	0.0001
	REP	2	3.2787696	1.6393848	12.95	0.0001
	LEVEL	2	7.7546088	3.8773044	10.33	0.0003
	CULT	34	19.760103	0.5811795	4.59	0.0001
	Error	68	8.6075764	0.126582		
	Total	104	31.646449			
			R-Square	C.V.	R-MSE	Mean
			0.728008	21.1296	0.35578	1.6838166

8.2.4 ANOVA for Family K samples group

PPD-MARKER

DAY 0

Source	DF	SS	MS	F Value	Pr > F
Model	36	0.0006054	1.682E-05	1	0.488
REP	2	3.363E-05	1.682E-05	1	0.3732
LEVEL	2	4.204E-05	2.102E-05	1.27	0.2946
CULT	34	0.0005717	1.682E-05	1	0.4868
Error	68	0.0011435	1.682E-05		
Total	104	0.0017489			

R-Square	C.V.	R-MSE	Mean
0.346154	1024.695	0.0041	0.0004002

DAY 1

Source	DF	SS	MS	F Value	Pr > F
Model	36	0.1917513	0.0053264	1.13	0.3232
REP	2	0.0144121	0.007206	1.53	0.2233
LEVEL	2	0.0022235	0.0011118	0.2	0.8172
CULT	34	0.1773393	0.0052159	1.11	0.3509
Error	68	0.3196974	0.0047014		
Total	104	0.5114487			

R-Squa	C.V.	R-MSE	Mean
0.37	166.7234	0.068567	0.0411262

DAY 2

Source	DF	SS	MS	F Value	Pr > F
Model	36	9.9256116	0.2757114	1.41	0.1114
REP	2	0.7769969	0.3884984	1.99	0.1452
LEVEL	2	1.4560098	0.7280049	3.03	0.0624
CULT	34	9.1486147	0.2690769	1.38	0.1319
Error	68	13.304823	0.1956592		
Total	104	23.230434			

R-Squa	C.V.	R-MSE	Mean
0.43	165.003	0.4423338	0.2680762

DAY 3

Source	DF	SS	MS	F Value	Pr > F
Model	36	35.070362	0.9741767	2.77	0.0001
REP	2	4.1443739	2.072187	5.88	0.0044
LEVEL	2	7.9403812	3.9701906	5.53	0.0087
CULT	34	30.925988	0.9095879	2.58	0.0004
Error	68	23.950825	0.352218		
Total	104	59.021188			

R-Squa	C.V.	R-MSE	Mean
0.59	86.0226	0.5934796	0.6899112

DAY 4

Source	DF	SS	MS	F Value	Pr > F
Model	36	79.373195	2.204811	1.7	0.0303
REP	2	8.4225733	4.2112867	3.24	0.0452
LEVEL	2	17.362812	8.6814058	5.18	0.0112
CULT	34	70.950622	2.086783	1.61	0.0488
Error	68	88.329492	1.2989631		
Total	104	167.70269			

R-Square	C.V.	R-MSE	Mean
0.473297	111.686	1.13972	1.0204687

Ln CURVE AREA

Source	DF	SS	MS	F Value	Pr > F
Model	36	17.976005	0.4993335	5.36	0.0001
REP	2	2.6404469	1.3202235	14.18	0.0001
LEVEL	2	5.7239266	2.8619633	9.53	0.0006
CULT	34	15.335558	0.4510458	4.85	0.0001
Error	68	6.3296357	0.0930829		
Total	104	24.305641			

R-Square	C.V.	R-MSE	Mean
0.739582	38.4829	0.30509	0.7928063

PHEN-CON

DAY 0

Source	DF	SS	MS	F Value	Pr > F
Model	36	1797.6229	49.933969	1.5	0.0764
REP	2	131.67301	65.836505	1.97	0.147
LEVEL	2	246.03127	123.01564	2.77	0.0776
CULT	34	1665.9499	48.998526	1.47	0.0896
Error	68	2269.6626	33.377391		
Total	104	4067.2855			

R-Square	C.V.	R-MSE	Mean
0.441971	33.90155	5.77732	17.041455

DAY 1

Source	DF	SS	MS	F Value	Pr > F
Model	36	1767.6362	49.101005	2.15	0.0035
REP	2	229.15722	114.57861	5.01	0.0094
LEVEL	2	84.285996	42.142998	0.94	0.4001
CULT	34	1527.637	44.930499	1.96	0.0094
Error	67	1533.056	22.881432		
Total	103	3300.6922			

R-Square	C.V.	R-MSE	Mean
0.535535	28.59016	4.78345	16.731119

DAY 2

Source	DF	SS	MS	F Value	Pr > F
Model	36	1697.5305	47.153625	1.41	0.1089
REP	2	43.466748	21.733374	0.65	0.5242
LEVEL	2	388.29732	194.14866	4.91	0.0138
CULT	34	1654.0637	48.648933	1.46	0.093
Error	68	2266.8144	33.335505		
Total	104	3964.3448			

R-Square	C.V.	R-MSE	Mean
0.4282	31.88896	5.77369	18.105611

DAY 3

Source	DF	SS	MS	F Value	Pr > F
Model	36	1781.4579	49.484941	1.99	0.0072
REP	2	503.91962	251.95981	10.14	0.0001
LEVEL	2	44.999598	22.499799	0.58	0.5634
CULT	34	1277.5383	37.574655	1.51	0.074
Error	68	1689.2803	24.842357		
Total	104	3470.7382			

R-Square	C.V.	R-MSE	Mean
0.513279	25.27579	4.98421	19.719308

DAY 4

Source	DF	SS	MS	F Value	Pr > F
Model	36	2291.6789	63.657747	1.5	0.0763
REP	2	397.77876	198.88938	4.68	0.0125
LEVEL	2	108.44897	54.224485	0.97	0.3893
CULT	34	1893.9001	55.702945	1.31	0.1715
Error	68	2892.8113	42.541343		
Total	104	5184.4902			

R-Square	C.V.	R-MSE	Mean
0.442026	32.20725	6.52237	20.251258

Ln CURVE AREA

Source	DF	SS	MS	F Value	Pr > F
Model	36	4.12783	0.114662	2.54	0.0005
REP	2	0.5227977	0.2613989	5.79	0.0048
LEVEL	2	0.2744044	0.1372022	1.32	0.2818
CULT	34	3.6050323	0.1060304	2.35	0.0014
Error	68	3.0701709	0.0451496		
Total	104	7.1980009			

R-Square	C.V.	R-MSE	Mean
0.573469	4.978067	0.21248	4.2684093

8.3 REGWQ GROUPING

Data were analysed using ANOVA and mean values were separated by REGWQ. The REGWQ option performs the Ryan-Einot-Gabriel-Welsch multiple range test on all main effect means in the MEANS statement using the GLM procedure of SAS. Means with the same letter are not statistically different. In the following tables, level refers to the PPD level group of cultivars.

The variables considered for the analysis were the measurements of secondary metabolites or enzymatic activity at each day during the time course or the natural logarithm of the area under the curve (AUTC).

8.3.1 REGWQ grouping by PPD susceptibility level

8.3.1.1 REGWQ grouping by PPD susceptibility level for Bath samples group

ESCULIN				ESCULETIN				SCOPOLIN				SCOPOLETIN			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	1.82	m		A	7.904	h		A	10.566	m		A	27.76	m
DAY 0	A	1.118	h	DAY 0	A	5.419	l	DAY 0	A	7.939	h	DAY 0	A	26.75	h
	A	0.428	l		A	4.303	m		A	1.618	l		A	24.22	l
	A	4.809	h		A	16.38	h		A	10.624	h		A	57.15	h
DAY 1	A	1.715	l	DAY 1	A	3.48	l	DAY 1	A	9.188	m	DAY 1	A	37.49	m
	A	1.423	m		A	3.28	m		A	7.623	l		A	26.43	l
	A	11.45	h		A	12.708	l		A	45.63	m		A	63.11	h
DAY 2	A	9.52	l	DAY 2	A	12.551	h	DAY 2	B	35.44	h	DAY 2	A	50.51	m
	A	2.23	m		A	1.967	m		B	13.32	l		A	20.73	l
	A	7.9	l		A	26.574	l		A	58.38	m		A	31.98	m
DAY 3	A	5.196	h	DAY 3	A	5.178	h	DAY 3	A	31	h	DAY 3	A	25.29	h
	A	3.783	m		A	3	m		A	13.27	l		A	2.96	l
	A	7.853	h		A	29.64	l		A	52.69	m		A	33.51	h
DAY 4	A	5.349	l	DAY 4	A	15.08	h	DAY 4	A	50.53	h	DAY 4	A	23.04	l
	A	3.593	m		A	3.59	m		A	16.05	l		A	17.95	m
	A	34.2	l		A	19.76	l		A	60.16	h		A	54.31	h
DAY 5	A	21.06	h	DAY 5	A	16.67	h	DAY 5	A	52.95	l	DAY 5	A	27.03	l
	A	4.8	m		A	5.33	m		A	44.23	m		A	20.02	m
	A	24.09	h		A	19.26	l		A	130.22	l		A	124.54	l
DAY 6	A	18.1	l	DAY 6	A	11.614	h	DAY 6	A	33.18	m	DAY 6	A	61.05	h
	A	3.13	m		A	4.239	m		A	23.59	h		A	30.49	m
	A	25.32	h		A	8.17	l		A	55.9	l		A	56.15	h
DAY 7	A	24.61	l	DAY 7	B	7.941	h	DAY 7	A	49.83	m	DAY 7	A	34.44	l
	A	4.12	m		B	2.914	m		A	45.22	h		A	29.43	m
Ln	A	4.5388	l	Ln	A	4.2618	l	Ln	A	5.6104	l	Ln	A	5.6723	h
CURVE	A	3.7671	h	CURVE	A	4.0608	h	CURVE	A	5.1832	h	CURVE	A	5.5993	l
AREA	A	3.0651	m	AREA	A	3.1759	m	AREA	A	5.0701	m	AREA	A	5.3971	m

GALLOCATECHIN				CATECHIN				CATE-GALLATE			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	3.323	m		A	5.474	m		A	4.504	m
DAY 0	A	2.369	h	DAY 0	B	2.628	h	DAY 0	A	1.724	h
	A	0.021	l		B	1.262	l		A	0.764	l
	A	5.658	m		A	13.14	h		A	2.465	h
DAY 1	A	2.006	l	DAY 1	A	6.14	m	DAY 1	A	2.377	m
	A	0.611	h		A	0.46	l		A	0.327	l
	A	19.12	h		A	11.247	h		A	5.249	h
DAY 2	A	2.34	m	DAY 2	A	5.31	m	DAY 2	A	1.661	m
	A	0.45	l		A	1.555	l		A	0.678	l
	A	24.25	h		A	10.401	m		A	3.593	h
DAY 3	A	16.66	m	DAY 3	A	6.053	h	DAY 3	B	1.048	m
	A	10.18	l		A	0.889	l		B	0.777	l
	A	120.33	l		A	45.946	l		A	10.41	l
DAY 4	A	49.77	h	DAY 4	A	9.749	h	DAY 4	A	4.405	h
	A	17.02	m		A	6.741	m		A	2.36	m
	A	90.99	h		A	30.02	l		A	7.896	l
DAY 5	A	70.06	l	DAY 5	A	29.17	h	DAY 5	A	6.626	h
	A	52.02	m		A	12.01	m		A	1.82	m
	A	368.6	l		A	128.85	l		A	12.479	l
DAY 6	A	149.8	h	DAY 6	A	47.16	h	DAY 6	A	11.713	h
	A	39.7	m		A	27.65	m		A	2.265	m
	A	397.8	l		A	84.68	l		A	25.297	l
DAY 7	A	149.7	h	DAY 7	A	25.26	h	DAY 7	A	10.777	h
	A	20.7	m		A	19.55	m		A	9.156	m
Ln	A	6.734	l	Ln	A	5.3613	l	Ln	A	3.934	l
CURVE	A	5.136	h	CURVE	A	4.299	h	CURVE	A	3.2803	h
AREA	A	4.761	m	AREA	A	4.1076	m	AREA	A	3.0288	m

8.3.1.2 *REGWQ grouping by PPD susceptibility level for June 1999 samples group*

	POX				PPO				CAT				PAL			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	0.0028333	h		A	0.0008167	h		A	0.0003167	m		A	0.5334	h
DAY 0	A	0.0015	l	DAY 0	A	0.00035	m	DAY 0	A	0.0001667	h	DAY 0	A	0.2614	l
	A	0.0004167	m		A	0.0001333	l		A	0.0000333	l		A	0.203	m
	A	0.003283	h		A	0.002883	h		A	0.00015	h		A	0.4101	h
DAY 1	A	0.001217	l	DAY 1	A	0.001033	m	DAY 1	A	0.00013333	l	DAY 1	A	0.352	m
	A	0.001	m		A	0.00055	l		A	0.00013333	m		A	0.1584	l
	A	0.00295	h		A	0.0026	h		A	0.0003	m		A	0.5477	h
DAY 2	A	0.001517	l	DAY 2	A	0.000967	l	DAY 2	A	0.0001833	h	DAY 2	A	0.5145	m
	A	0.000817	m		A	0.000683	m		A	0.0001167	l		A	0.1824	l
	A	0.003233	h		A	0.002717	h		A	0.0003	m		A	0.9997	h
DAY 3	A	0.002033	l	DAY 3	A	0.001133	l	DAY 3	A	0.0002	h	DAY 3	A	0.6074	m
	A	0.001117	m		A	0.000233	m		A	0.00008333	l		A	0.3847	l
	A	0.005067	m		A	0.00388	h		A	0.00041667	m		A	2.2257	h
DAY 4	A	0.004017	h	DAY 4	A	0.001783	m	DAY 4	A	0.00016667	h	DAY 4	A	2.1633	m
	A	0.0027	l		A	0.000783	l		A	0.00011667	l		A	0.9266	l
	A	0.00725	m		A	0.00805	h		A	0.0002667	m		A	3.391	h
DAY 5	A	0.00705	h	DAY 5	A	0.000817	m	DAY 5	A	0.0002333	l	DAY 5	A	2.2124	m
	A	0.00325	l		A	0.000633	l		A	0.0000667	h		A	1.1882	l
	A	0.007733	m		A	0.0068	h		A	0.0002167	m		A	2.7621	h
DAY 6	A	0.00605	h	DAY 6	A	0.001267	m	DAY 6	A	0.0002167	l	DAY 6	A	1.9206	m
	A	0.006017	l		A	0.000767	l		A	0.0001667	h		A	1.8222	l
	A	0.011367	h		A	0.0108	h		A	0.0002333	l		A	5.461	h
DAY 7	A	0.0096	m	DAY 7	A	0.00265	m	DAY 7	A	0.0001333	h	DAY 7	A	2.444	m
	A	0.005167	l		A	0.0011	l		A	0.0001333	m		A	1.98	l
Ln	A	0.033	h	Ln	A	0.03107	h	Ln	A	0.0018563	m	Ln	A	2.5694	h
CURVE	A	0.02754	m	CURVE	A	0.00727	m	CURVE	A	0.0010824	h	CURVE	A	2.1857	m
AREA	A	0.01983	l	AREA	A	0.00542	l	AREA	A	0.0010326	l	AREA	A	1.8486	l

	GLUCANASE				CHITINASE				SCOPOLETIN				SCOPOLIN			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	8.044	m		A	1.961	m		A	8.044	m		A	1.961	m
DAY 0	A	3.316	l	DAY 0	A	1.637	l	DAY 0	A	3.316	l	DAY 0	A	1.637	l
	A	3.066	h		A	1.43	h		A	3.066	h		A	1.43	h
	A	16.232	l		A	8.329	l		A	16.232	l		A	8.329	l
DAY 1	A	10.893	m	DAY 1	A	7.507	h	DAY 1	A	10.893	m	DAY 1	A	7.507	h
	A	8.806	h		A	3.879	m		A	8.806	h		A	3.879	m
	A	32	h		A	19.579	h		A	32	h		A	19.579	h
DAY 2	A	20.9	l	DAY 2	A	12.118	l	DAY 2	A	20.9	l	DAY 2	A	12.118	l
	A	20.14	m		A	8.222	m		A	20.14	m		A	8.222	m
	A	25.24	h		A	47.35	h		A	25.24	h		A	47.35	h
DAY 3	A	21.07	m	DAY 3	A	23.08	m	DAY 3	A	21.07	m	DAY 3	A	23.08	m
	A	19.01	l		A	16.06	l		A	19.01	l		A	16.06	l
	A	26.67	h		A	36.788	h		A	26.67	h		A	36.788	h
DAY 4	A	22.22	m	DAY 4	A	27.857	m	DAY 4	A	22.22	m	DAY 4	A	27.857	m
	A	17.75	l		A	16.496	l		A	17.75	l		A	16.496	l
	A	37.95	h		A	42.41	h		A	37.95	h		A	42.41	h
DAY 5	A	24.61	m	DAY 5	A	32.36	m	DAY 5	A	24.61	m	DAY 5	A	32.36	m
	A	10.95	l		A	11.15	l		A	10.95	l		A	11.15	l
	A	25.34	m		A	39.55	h		A	25.34	m		A	39.55	h
DAY 6	A	23.31	h	DAY 6	A	30.04	m	DAY 6	A	23.31	h	DAY 6	A	30.04	m
	A	11.99	l		A	25.43	l		A	11.99	l		A	25.43	l
	A	20.3	h		A	25.104	m		A	20.3	h		A	25.104	m
DAY 7	A	16.05	l	DAY 7	A	25.098	h	DAY 7	A	16.05	l	DAY 7	A	25.098	h
	A	11.92	m		A	15.828	l		A	11.92	m		A	15.828	l
Ln	A	5.0735	h	Ln	A	5.2865	h	Ln	A	5.0735	h	Ln	A	5.2865	h
CURVE	A	4.7438	m	CURVE	A	4.9016	m	CURVE	A	4.7438	m	CURVE	A	4.9016	m
AREA	A	4.6636	l	AREA	A	4.5315	l	AREA	A	4.6636	l	AREA	A	4.5315	l

8.3.1.2 REGWQ grouping by PPD susceptibility level for June 1999 samples group

PPD-MARKER				PHEN-CON				TANNIN			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	0	h		A	32.14	h		A	1536.2	h
DAY 0	B	0	l	DAY 0	A	27.06	m	DAY 0	A	1024.1	m
	C	0	m		A	21.31	l		A	817.4	l
	A	0.07105	l		A	26.33	h		A	1536	h
DAY 1	A	0	h	DAY 1	A	23.93	m	DAY 1	A	1382.7	m
	A	0	m		A	20.1	l		A	662.6	l
	A	0.2392	l		A	23.79	h		A	977.9	m
DAY 2	A	0.0723	h	DAY 2	A	22.65	m	DAY 2	A	929.8	h
	A	0.0551	m		A	16.68	l		A	636.8	l
	A	1.568	m		A	28.4	h		A	1100.7	h
DAY 3	A	0.531	l	DAY 3	A	21.46	m	DAY 3	A	687.1	m
	A	0.485	h		A	18.07	l		A	660.8	l
	A	2.099	m		A	27.36	h		A	1544.2	h
DAY 4	A	0.751	h	DAY 4	A	23.96	m	DAY 4	A	807.8	m
	A	0.378	l		A	15.65	l		A	648	l
	A	1.4469	h		A	32.051	h		A	1491.9	h
DAY 5	A	0.6629	l	DAY 5	A	20.663	m	DAY 5	A	785.8	m
	A	0.4478	m		A	15.881	l		A	718.9	l
	A	1.2319	l		A	27.79	h		A	1160.1	h
DAY 6	A	1.1715	m	DAY 6	A	26.81	m	DAY 6	A	750.9	m
	A	1.1418	h		A	13.03	l		A	714.1	l
	A	1.5697	l		A	29.245	h		A	1908	h
DAY 7	A	1.3403	h	DAY 7	A	16.985	m	DAY 7	A	772	m
	A	0.5169	m		A	15.302	l		A	747.2	l
Ln	A	1.6561	h	Ln	A	5.2092	h	Ln	A	9.0398	h
CURVE	A	1.5372	l	CURVE	A	4.9605	m	CURVE	A	8.7413	m
AREA	A	1.432	m	AREA	A	4.7732	l	AREA	A	8.4776	l

8.3.1.3 REGWQ grouping by PPD susceptibility level for December 1999 samples

POX				SCP-POX				PPO			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	0.0015673	l		A	0.007232	m		A	0.0001183	m
DAY 0	A	0.0013647	h	DAY 0	A	0.006691	l	DAY 0	A	0.00010428	l
	A	0.0011373	m		A	0.004468	h		A	0.00004177	h
	A	0.0020206	l		A	0.012002	m		A	0.0009973	h
DAY 1	A	0.0019862	m	DAY 1	A	0.007455	h	DAY 1	A	0.0008655	l
	A	0.0018333	h		A	0.004996	l		A	0.0000842	m
	A	0.0023929	l		A	0.014199	m		A	0.0016182	h
DAY 2	A	0.0020695	h	DAY 2	A	0.014072	h	DAY 2	A	0.0014144	l
	A	0.0017863	m		A	0.007312	l		A	0.0003089	m
	A	0.0018251	l		A	0.022276	h		A	0.0019464	l
DAY 3	A	0.0015413	h	DAY 3	A	0.013639	m	DAY 3	A	0.0018399	h
	A	0.0013688	m		A	0.012448	l		A	0.0004251	m
	A	0.0015043	h		A	0.02945	h		A	0.0020505	h
DAY 4	A	0.001385	l	DAY 4	A	0.02199	m	DAY 4	A	0.00176	l
	A	0.0013379	m		A	0.01752	l		A	0.0007204	m
Ln	A	0.007675	l	Ln	A	0.05879	h	Ln	A	0.005482	h
CURVE	A	0.006854	h	CURVE	A	0.0525	m	CURVE	A	0.005136	l
AREA	A	0.006357	m	AREA	A	0.03611	l	AREA	A	0.001236	m

CAT				SCOPOLETIN				SCOPOLIN			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	0.000428	m		A	10.171	m		A	4.542	m
DAY 0	A	0.0003849	h	DAY 0	A	7.062	h	DAY 0	A	3.13	l
	A	0.0003834	l		A	5.316	l		A	1.387	h
	A	0.000507	m		A	39.248	h		A	19.16	h
DAY 1	A	0.0004965	h	DAY 1	A	16.865	l	DAY 1	A	12.11	m
	A	0.0003639	l		A	16.549	m		A	10.22	l
	A	0.0006557	h		A	36.97	h		A	27.63	h
DAY 2	A	0.0005608	l	DAY 2	A	36.77	m	DAY 2	A	25.94	l
	A	0.0005255	m		A	28.72	l		A	22.69	m
	A	0.0005485	l		A	41.58	h		A	53.47	h
DAY 3	A	0.0005462	h	DAY 3	A	38.7	m	DAY 3	A	35.69	m
	A	0.0005312	m		A	31.75	l		A	23.99	l
	A	0.0006486	l		A	37.84	m		A	51.21	h
DAY 4	A	0.0005101	m	DAY 4	A	31.67	l	DAY 4	A	30.73	l
	A	0.0004731	h		A	30.89	h		A	30.03	m
Ln	A	0.002125	h	Ln	A	4.8924	h	Ln	A	4.6888	h
CURVE	A	0.0020303	m	CURVE	A	4.6018	m	CURVE	A	4.4588	m
AREA	A	0.0019869	l	AREA	A	4.5317	l	AREA	A	4.2538	l

8.3.1.3 REGWQ grouping by PPD susceptibility level for December 1999 samples

ESCULIN				PPD-MARKER				PHEN-CON			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
	A	0.04099	h		A	0	h		A	28.76	m
DAY 0	A	0	l	DAY 0	B	0	l	DAY 0	A	28.57	l
	A	0	m		C	0	m		A	27.9	h
	A	0.4395	h		A	0.10065	h		A	33.048	m
DAY 1	A	0.2855	l	DAY 1	A	0.0322	m	DAY 1	A	29.324	l
	A	0.0815	m		A	0.01746	l		A	23.449	h
	A	2.565	h		A	0.86	h		A	35.612	m
DAY 2	A	0.793	l	DAY 2	A	0.3707	m	DAY 2	A	27.717	l
	A	0.273	m		A	0.0823	l		A	24.202	h
	A	5.642	h		A	2.1517	h		A	30.561	l
DAY 3	A	1.345	m	DAY 3	B	0.7544	m	DAY 3	A	27.758	m
	A	0.923	l		B	0.6426	l		A	26.4	h
	A	3.52	h		A	2.1287	h		A	34.552	m
DAY 4	A	2.952	m	DAY 4	A	1.9498	m	DAY 4	A	33.144	l
	A	1.299	l		A	1.0203	l		A	26.625	h
Ln	A	2.0998	h	Ln	A	1.5208	h	Ln	A	4.819	m
CURVE	A	1.188	l	CURVE	B A	1.0157	m	CURVE	A	4.7197	l
AREA	A	1.1222	m	AREA	B	0.6944	l	AREA	A	4.6098	h

8.3.1.4 REGWQ grouping by PPD susceptibility level for Family K samples group

	POX				SCP-POX				PPO				SCOPOLETIN			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
DAY 0	A	0.00139233	m	DAY 0	A	0.004893	m	DAY 0	A	0.00011367	l	DAY 0	A	9.006	h
	A	0.00137867	h		A	0.004315	l		A	0.00009533	m		A	7.175	m
	B	0.00115967	l		A	0.004029	h		A	0.00008267	h		A	5.763	l
DAY 1	A	0.002168	h	DAY 1	A	0.0057196	h	DAY 1	A	0.0007263	l	DAY 1	A	21.94	h
	A	0.001993	m		A	0.0056823	m		A	0.0007047	m		B A	17.991	m
	A	0.001455	l		A	0.0050863	l		A	0.0005713	h		B	15.74	l
DAY 2	A	0.2459	h	DAY 2	A	0.012727	l	DAY 2	A	0.001413	m	DAY 2	A	52.021	h
	A	0.002	m		A	0.011639	m		A	0.0013107	l		B	33.276	m
	A	0.0015	l		A	0.011346	h		A	0.0009947	h		B	26.513	l
DAY 3	A	0.0021791	h	DAY 3	A	0.025289	h	DAY 3	A	0.0015497	m	DAY 3	A	50.411	h
	B A	0.001879	m		A	0.022901	m		A	0.0014	l		B	36.996	m
	B	0.0015023	l		B	0.015678	l		A	0.0012027	h		B	27.578	l
DAY 4	A	0.0020267	h	DAY 4	A	0.036352	h	DAY 4	A	0.001863	m	DAY 4	A	40.466	h
	B A	0.0017227	m		A	0.035337	m		A	0.0015477	l		B A	34.395	m
	B	0.0014527	l		A	0.028196	l		A	0.0012673	h		B	25.088	l
Ln	A	0.06292	h	Ln	A	0.060465	h	Ln	A	0.0046335	m	Ln	A	4.9593	h
CURVE	A	0.00741	m	CURVE	A	0.058377	m	CURVE	A	0.0042531	l	CURVE	B	4.6251	m
AREA	A	0.00573	l	AREA	A	0.048323	l	AREA	A	0.0034358	h	AREA	B	4.3883	l

	SCOPOLIN				ESCULIN				PPD-MARKER				PHEN-CON			
	GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL		GROUP	MEAN	LEVEL
DAY 0	A	4.521	l	DAY 0	A	0.13926	l	DAY 0	A	0.0014007	l	DAY 0	A	19.296	l
	A	3.614	h		A	0.08373	h		A	0	m		A	16.677	h
	A	3.505	m		A	0.06029	m		A	0	h		A	15.333	m
DAY 1	A	15.543	l	DAY 1	A	0.5919	m	DAY 1	A	0.04634	m	DAY 1	A	18.227	l
	A	13.536	h		A	0.4945	h		A	0.04214	h		A	16.307	m
	A	12.61	m		A	0.3196	l		A	0.03439	l		A	16.049	h
DAY 2	A	35.618	m	DAY 2	A	1.9391	m	DAY 2	A	0.4074	m	DAY 2	A	20.638	l
	A	34.781	h		A	1.6862	h		B A	0.2878	h		B A	18.121	h
	A	28.635	l		B	0.8247	I		B	0.0991	l		B	15.55	m
DAY 3	A	48.834	h	DAY 3	A	2.7554	m	DAY 3	A	0.9474	m	DAY 3	A	20.714	l
	A	46.819	m		A	2.366	h		A	0.801	h		A	19.495	h
	B	32.616	l		B	1.0936	l		B	0.2658	l		A	19.06	m
DAY 4	A	53.688	m	DAY 4	A	2.9397	m	DAY 4	A	1.3618	m	DAY 4	A	21.857	l
	A	53.598	h		A	2.8496	h		A	1.2168	h		A	19.644	h
	B	38.056	l		B	1.1485	l		B	0.3846	l		A	19.556	m
Ln	A	4.7829	h	Ln	A	1.8715	m	Ln	A	0.9581	m	Ln	A	4.34537	l
CURVE	A	4.7617	m	CURVE	A	1.8449	h	CURVE	A	0.9284	h	CURVE	A	4.25272	h
AREA	A	4.5019	l	AREA	B	1.2545	l	AREA	B	0.4242	l	AREA	A	4.21498	m

8.3.2 REGWQ grouping by cultivar

8.3.2.1 REGWQ grouping by cultivar for Bath samples group

ESCULIN								ESCULETIN							
	GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR
DAY 0	A	2.602	MNGA2	DAY 4	A	16.254	MDOM5	DAY 0	A	19.89	SM985-9	DAY 4	A	40.15	SM985-9
	A	2.584	MCOL22		A	8.702	CM7033-3		A	6.03	MDOM5		A	29.64	MBRA337
	A	1.375	MDOM5		A	5.349	MBRA337		A	5.42	MBRA337		A	10.09	MDOM5
	A	1.039	MNGA1		A	4.338	MCOL22		A	4.7	MNGA2		A	6.61	MNGA1
	A	0.428	MBRA337		A	4.274	MNGA2		A	3.9	MNGA1		A	6.6	CM7033-3
	A	0.286	SM985-9		A	2.913	MNGA1		A	3.43	MCOL22		A	3.46	MCOL22
	A	0.226	CM7033-3		A	2.118	SM985-9		A	2.27	CM7033-3		A	0.56	MNGA2
DAY 1	A	11.47	MDOM5	DAY 8	A	43.97	MDOM5	DAY 1	A	35.52	CM7033-3	DAY 8	A	40.25	SM985-9
	A	6.2	MCOL22		A	34.2	MBRA337		A	10.91	MCOL22		A	19.76	MBRA337
	A	1.828	MNGA2		A	22.25	SM985-9		A	10.62	SM985-9		A	11.47	MDOM5
	A	1.715	MBRA337		A	16.53	CM7033-3		A	8.48	MDOM5		A	11.4	CM7033-3
	A	1.468	CM7033-3		A	4.95	MNGA1		A	4.08	MNGA1		A	8.88	MNGA1
	A	1.018	MNGA1		A	4.66	MNGA2		A	3.48	MBRA337		A	3.57	MCOL22
	A	0.098	SM985-9		A	1.47	MCOL22		A	2.48	MNGA2		A	1.77	MNGA2
DAY 2	A	30.5	MDOM5	DAY 6	A	45.87	MDOM5	DAY 2	A	22.34	CM7033-3	DAY 6	A	19.26	MBRA337
	A	9.52	MBRA337		A	45.43	SM985-9		A	16.77	SM985-9		A	15.267	MDOM5
	A	7.22	CM7033-3		A	18.1	MBRA337		A	12.71	MBRA337		A	14.851	SM985-9
	A	5.3	MCOL22		A	4.3	CM7033-3		A	6.39	MCOL22		A	14.464	CM7033-3
	A	3.26	MNGA2		A	3.73	MNGA2		A	4.7	MDOM5		A	4.629	MNGA2
	A	2.77	SM985-9		A	2.53	MNGA1		A	2.9	MNGA1		A	3.849	MNGA1
	A	1.2	MNGA1		A	0.78	MCOL22		A	1.04	MNGA2		A	1.872	MCOL22
DAY 3	A	12.775	MDOM5	DAY 7	A	86.55	MDOM5	DAY 3	A	26.57	MBRA337	DAY 7	A	14.27	MDOM5
	A	7.9	MBRA337		A	24.61	MBRA337		A	8.95	SM985-9		A	8.17	MBRA337
	A	5.437	CM7033-3		A	10.66	SM985-9		A	5.83	MDOM5		A	6.214	MCOL22
	A	4.029	MNGA1		A	4.89	MNGA2		A	4.64	MNGA1		A	6.108	CM7033-3
	A	3.537	MNGA2		A	3.34	MNGA1		A	3.44	CM7033-3		A	5.172	SM985-9
	A	1.847	MCOL22		A	2.86	CM7033-3		A	2.5	MCOL22		A	3.825	MNGA1
	A	0.723	SM985-9		A	1.21	MCOL22		A	1.36	MNGA2		A	2.003	MNGA2
Ln CURVE AREA	A	4.72	MDOM5					Ln CURVE AREA	A	4.5482	SM985-9				
	A	4.5388	MBRA337						A	4.2618	MBRA337				
	A	3.789	SM985-9						A	4.191	MDOM5				
	A	3.5468	CM7033-3						A	3.9234	CM7033-3				
	A	3.2508	MNGA2						A	3.5807	MCOL22				
	A	3.0124	MCOL22						A	3.4741	MNGA1				
	A	2.8795	MNGA1						A	2.8778	MNGA2				

8.3.2.1 REGWQ grouping by cultivar for Bath samples group

SCOPOLIN

	GROUP	MEAN	CULTIVAR
DAY 0	A	19.404	MDOM5
	A	16.822	MNGA2
	A	9.286	SM985-9
	A	4.31	MNGA1
	A	1.815	MCOL22
	A	1.618	MBRA337
	A	1.252	CM7033-3
DAY 1	A	17.361	MDOM5
	A	14.926	MNGA2
	A	13.229	MCOL22
	A	7.623	MBRA337
	A	6.796	CM7033-3
	A	5.109	SM985-9
	A	3.451	MNGA1
DAY 2	A	75.01	MNGA2
	A	68.68	MCOL22
	A	25.13	CM7033-3
	A	24.73	MDOM5
	A	23.23	SM985-9
	A	16.25	MNGA1
	A	13.32	MBRA337
DAY 3	A	70.41	MNGA2
	A	58.42	CM7033-3
	A	46.36	MNGA1
	A	43.26	MDOM5
	A	20.18	MCOL22
	A	13.27	MBRA337
	A	2.15	SM985-9
Ln CURVE AREA	A	5.8659	MNGA2
	A	5.7168	CM7033-3
	A	5.6526	MDOM5
	A	5.6104	MBRA337
	A	4.7314	SM985-9
	A	4.632	MCOL22
	A	4.2742	MNGA1

	GROUP	MEAN	CULTIVAR
DAY 4	A	103.91	MDOM5
	A	77.38	MNGA2
	A	70.72	CM7033-3
	A	28	MNGA1
	A	23.26	MCOL22
	A	16.05	MBRA337
	A	4.22	SM985-9
DAY 8	A	187.75	CM7033-3
	A	52.95	MBRA337
	A	48.28	MNGA2
	A	41.95	MDOM5
	A	40.19	MNGA1
	A	7.21	SM985-9
	A	3.73	MCOL22
DAY 6	A	130.22	MBRA337
	B A	51.11	MNGA2
	B A	46.65	MDOM5
	B	29.71	CM7033-3
	B	16.47	SM985-9
	B	15.24	MNGA1
	B	1.51	MCOL22
DAY 7	A	96.32	MNGA2
	A	88.8	MDOM5
	A	63.78	SM985-9
	A	55.9	MBRA337
	A	24.47	CM7033-3
	A	3.84	MCOL22
	A	3.35	MNGA1

SCOPOLETIN

	GROUP	MEAN	CULTIVAR
DAY 0	A	45.09	MDOM5
	A	34.87	MNGA2
	A	32.69	SM985-9
	A	24.51	MCOL22
	A	24.22	MBRA337
	A	20.64	MNGA1
	A	4.71	CM7033-3
DAY 1	A	99.72	MCOL22
	A	88.21	MDOM5
	A	41.19	MNGA2
	A	33.78	MNGA1
	A	26.43	MBRA337
	A	20.84	SM985-9
	A	19.86	CM7033-3
DAY 2	A	90.3	MDOM5
	A	81.17	CM7033-3
	A	62.88	MCOL22
	A	53.03	MNGA1
	A	47.99	MNGA2
	A	20.73	MBRA337
	A	18.11	SM985-9
DAY 3	A	48.52	MDOM5
	A	47.11	MNGA1
	A	31.56	MCOL22
	A	16.86	MNGA2
	A	15.21	CM7033-3
	A	5.86	SM985-9
	A	2.96	MBRA337
Ln CURVE AREA	A	6.3037	MDOM5
	A	5.6627	SM985-9
	A	5.6081	MCOL22
	A	5.5993	MBRA337
	A	5.4502	MNGA1
	A	5.3441	MNGA2
	A	5.1149	CM7033-3

	GROUP	MEAN	CULTIVAR
DAY 4	A	49.98	MDOM5
	A	43.34	MCOL22
	A	25.24	MNGA1
	A	24.94	CM7033-3
	A	23.04	MBRA337
	A	15.79	SM985-9
	A	10.66	MNGA2
DAY 8	A	83.62	MDOM5
	A	56.42	SM985-9
	A	40.44	CM7033-3
	A	36.77	MCOL22
	A	27.03	MBRA337
	A	20.93	MNGA2
	A	19.12	MNGA1
DAY 6	A	124.54	MBRA337
	A	123.95	MDOM5
	A	63.5	SM985-9
	A	52.07	CM7033-3
	A	38.39	MNGA1
	A	22.59	MNGA2
	A	4.68	MCOL22
DAY 7	A	112.21	MDOM5
	A	86.43	SM985-9
	A	34.44	MBRA337
	A	31.49	MNGA1
	A	27.36	MNGA2
	A	17.24	CM7033-3
	A	8.73	MCOL22

8.3.2.1 *REGWQ grouping by cultivar for Bath samples group*

(+)-GALLOCATECHIN								(+)-CATECHIN							
	GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR
DAY 0	A	8.704	SM985-9	DAY 4	A	135.59	CM7033-3	DAY 0	A	7.814	MNGA1	DAY 4	A	45.95	MBRA337
	A	6.57	MNGA2		A	120.33	MBRA337		A	6.151	SM985-9		A	19.13	CM7033-3
	A	0.656	MDOM5		A	49	MDOM5		A	3.621	MDOM5		A	10.47	SM985-9
	A	0.08	CM7033-3		A	20.81	MNGA1		A	3.134	MNGA2		A	9.02	MDOM5
	A	0.075	MNGA1		A	13.22	MNGA2		A	1.262	MBRA337		A	8	MNGA2
	A	0.037	MCOL22		A	8.81	MCOL22		A	0.396	MCOL22		A	5.49	MNGA1
	A	0.021	MBRA337		A	5.69	SM985-9		A	0.343	CM7033-3		A	0.38	MCOL22
DAY 1	A	10.138	MNGA1	DAY 8	A	188.83	MDOM5	DAY 1	A	30.32	MCOL22	DAY 8	A	73.33	CM7033-3
	A	2.064	SM985-9		A	88.4	MNGA1		A	14.18	MDOM5		A	31.23	SM985-9
	A	2.006	MBRA337		A	74.23	CM7033-3		A	9.35	MNGA1		A	30.02	MBRA337
	A	1.179	MNGA2		A	70.06	MBRA337		A	6.73	SM985-9		A	14.89	MNGA2
	A	0.235	MDOM5		A	62.18	MCOL22		A	2.92	MNGA2		A	9.44	MDOM5
	A	0.074	MCOL22		A	38.72	SM985-9		A	1.35	CM7033-3		A	9.12	MNGA1
	A	0.07	CM7033-3		A	15.63	MNGA2		A	0.46	MBRA337		A	2.68	MCOL22
DAY 2	A	48.85	MDOM5	DAY 6	A	368.6	MBRA337	DAY 2	A	19.86	CM7033-3	DAY 6	A	128.85	MBRA337
	A	25.63	CM7033-3		A	358.6	MDOM5		A	19.42	MDOM5		A	113.64	SM985-9
	A	4.64	MNGA1		A	83.1	SM985-9		A	7.75	MNGA1		A	53.63	MNGA2
	A	1.85	SM985-9		A	81.8	MCOL22		A	2.93	SM985-9		A	39.72	MDOM5
	A	0.45	MBRA337		A	75.8	CM7033-3		A	2.87	MNGA2		A	35.19	CM7033-3
	A	0.15	MCOL22		A	69.4	MNGA2		A	2.77	MCOL22		A	1.66	MNGA1
	A	0.05	MNGA2		A	10	MNGA1		A	1.55	MBRA337		A	0.1	MCOL22
DAY 3	A	41.93	MDOM5	DAY 7	A	397.8	MBRA337	DAY 3	A	17.924	MNGA1	DAY 7	A	84.68	MBRA337
	A	31.27	MNGA1		A	368.1	MDOM5		A	14.153	CM7033-3		A	53.75	SM985-9
	A	28.17	SM985-9		A	143.6	SM985-9		A	4.505	MDOM5		A	38.94	MNGA2
	A	25.01	CM7033-3		A	79.2	MCOL22		A	4.121	SM985-9		A	26.07	MDOM5
	A	10.18	MBRA337		A	21.1	MNGA1		A	2.878	MNGA2		A	16.41	CM7033-3
	A	2.05	MNGA2		A	20.2	MNGA2		A	1.433	MCOL22		A	4.79	MCOL22
	A	1.91	MCOL22		A	7.8	CM7033-3		A	0.889	MBRA337		A	0.15	MNGA1
Ln CURVE AREA	A	6.734	MBRA337					Ln CURVE AREA	A	5.361	MBRA337				
	A	6.568	MDOM5						A	4.923	CM7033-3				
	A	5.555	SM985-9						A	4.62	SM985-9				
	A	5.09	CM7033-3						A	4.52	MDOM5				
	A	4.769	MNGA1						A	4.376	MNGA2				
	A	4.753	MNGA2						A	3.839	MNGA1				
	A	3.333	MCOL22						A	3.132	MCOL22				

8.3.2.1 REGWQ grouping by cultivar for Bath samples group

(+)-CATECHIN GALLATE							
	GROUP	MEAN	CULTIVAR		GROUP	MEAN	CULTIVAR
DAY 0	A	7.848	MNGA1	DAY 4	A	10.41	MBRA337
	A	2.087	MDOM5		A	6.252	MCOL22
	A	1.975	MCOL22		A	5.617	MDOM5
	A	1.437	CM7033-3		A	4.364	CM7033-3
	A	1.398	SM985-9		A	3.835	MNGA1
	A	1.159	MNGA2		A	1.386	SM985-9
	A	0.764	MBRA337		A	0.885	MNGA2
DAY 1	A	4.501	MDOM5	DAY 8	A	15.471	CM7033-3
	A	3.947	MNGA1		A	7.896	MBRA337
	A	2.704	MCOL22		A	5.741	MCOL22
	A	1.561	CM7033-3		A	4.623	MDOM5
	A	1.096	SM985-9		A	2.543	MNGA1
	A	0.806	MNGA2		A	1.098	MNGA2
	A	0.327	MBRA337		A	0.668	SM985-9
DAY 2	A	12.626	CM7033-3	DAY 6	A	26.31	CM7033-3
	A	4.546	MCOL22		A	12.48	MBRA337
	A	3.061	MDOM5		A	9.13	MDOM5
	A	2.851	MNGA1		A	8.7	MCOL22
	A	0.763	SM985-9		A	2.7	SM985-9
	A	0.678	MBRA337		A	2.26	MNGA1
	A	0.47	MNGA2		A	2.26	MNGA2
DAY 3	A	6.926	MDOM5	DAY 7	A	25.3	MBRA337
	A	4.421	CM7033-3		A	13.69	MCOL22
	A	1.632	MCOL22		A	13.2	MDOM5
	A	1.43	MNGA1		A	12.85	MNGA2
	A	1.393	SM985-9		A	9.81	CM7033-3
	A	0.777	MBRA337		A	6.41	SM985-9
	A	0.665	MNGA2		A	5.46	MNGA1
Ln CURVE AREA	A	3.934	MBRA337				
	A	3.6493	MDOM5				
	A	3.4133	MCOL22				
	A	3.4086	CM7033-3				
	A	3.2749	MNGA1				
	A	2.7826	MNGA2				
	A	2.6498	SM985-9				

8.3.2.2 *REGWQ grouping by cultivar for June 1999 samples group*

	POX			PPO			CAT			PAL		
	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR
DAY 0	A	0.003067	CM2177-2	A	0.0015333	CM2177-2	A	0.0005333	MNGA2	A	0.0009333	MNGA2
	A	0.0026	MCOL22	A	0.0004667	MNGA2	B	0.0002	MCOL22	A	0.0003667	MCOL22
	A	0.002233	MPER183	A	0.0002667	MPER183	C B	0.0001333	CM2177-2	A	0.0003667	MVEN77
	A	0.000767	MBRA12	A	0.0002333	MVEN77	C B	0.0001	MVEN77	A	0.0003	MPER183
	A	0.000767	MNGA2	A	0.0001	MCOL22	C B	6.667E-05	MPER183	A	0.0002	CM2177-2
	A	0.000067	MVEN77	A	0	MBRA12	C	0	MBRA12	A	0.0002	MBRA12
DAY 1	A	0.0054	CM2177-2	A	0.005333	CM2177-2	A	0.0002	CM2177-2	A	0.0027	MNGA2
	B	0.0019333	MPER183	B A	0.002033	MNGA2	A	0.0001667	MNGA2	B	0.0003667	CM2177-2
	B	0.0016333	MNGA2	B	0.0011	MPER183	A	0.0001667	MPER183	B	0.0003333	MBRA12
	B	0.0011667	MCOL22	B	0.000433	MCOL22	A	0.0001	MBRA12	B	0.0003333	MVEN77
	B	0.0005	MBRA12	B	0.000033	MVEN77	A	0.0001	MCOL22	B	0.0003	MPER183
	B	0.0003667	MVEN77	B	0	MBRA12	A	0.0001	MVEN77	B	0.0001667	MCOL22
DAY 2	A	0.0044	CM2177-2	A	0.0050333	CM2177-2	A	0.0004	MNGA2	A	0.0014	MNGA2
	B A	0.0022333	MPER183	B	0.0018	MPER183	A	0.0002	CM2177-2	A	0.0004	CM2177-2
	B A	0.0016	MNGA2	B	0.0009667	MNGA2	A	0.0002	MPER183	A	0.0003333	MCOL22
	B A	0.0015	MCOL22	B	0.0004	MVEN77	A	0.0002	MVEN77	A	0.0003333	MVEN77
	B	0.0008	MBRA12	B	0.0001667	MCOL22	A	0.0001667	MCOL22	A	0.0003	MPER183
	B	0.0000333	MVEN77	B	0.0001333	MBRA12	A	0.0000333	MBRA12	A	0.0002	MBRA12
DAY 3	A	0.0048667	CM2177-2	A	0.0046667	CM2177-2	A	0.0004	MNGA2	A	0.0006333	MNGA2
	B A	0.0032667	MPER183	B	0.0022333	MPER183	A	0.0002333	CM2177-2	A	0.0003333	CM2177-2
	B C	0.0016	MCOL22	B	0.0007667	MCOL22	A	0.0002	MVEN77	A	0.0003333	MPER183
	B C	0.0013333	MVEN77	B	0.0003333	MVEN77	A	0.0001667	MCOL22	A	0.0003	MVEN77
	B C	0.0009	MNGA2	B	0.0001333	MNGA2	A	0.0001333	MPER183	A	0.0003	MCOL22
	C	0.0008	MBRA12	B	0.0000333	MBRA12	A	0.0000333	MBRA12	A	0.0002667	MBRA12
DAY 4	A	0.0067333	MNGA2	A	0.006133	CM2177-2	A	0.0005	MNGA2	A	0.0010333	MNGA2
	A	0.0054667	CM2177-2	A	0.0028	MNGA2	B A	0.0003333	MVEN77	A	0.0006333	MCOL22
	C	0.0042667	MPER183	A	0.001533	MPER183	B A	0.0002333	CM2177-2	A	0.0004333	CM2177-2
	C	0.0034	MVEN77	A	0.000767	MVEN77	B A	0.0001667	MPER183	A	0.0004333	MVEN77
	C	0.0025667	MCOL22	A	0.0005	MCOL22	B	0.0001	MCOL22	A	0.0003667	MPER183
		0.0011333	MBRA12	A	0.000033	MBRA12	B	0.0000667	MBRA12	A	0.0003	MBRA12
DAY 5	A	0.012267	CM2177-2	A	0.014433	CM2177-2	A	0.0003667	MPER183	A	0.0008667	CM2177-2
	A	0.0104	MNGA2	B	0.001667	MCOL22	A	0.0003667	MNGA2	A	0.0008333	MNGA2
	B	0.005067	MPER183	B	0.001433	MNGA2	B A	0.0001667	MVEN77	B A	0.0005333	MCOL22
	B	0.0041	MVEN77	B	0.0012	MPER183	B	0.0001	MCOL22	B A	0.0004667	MVEN77
	B	0.001833	MCOL22	B	0.0002	MVEN77	B	0.0001	MBRA12	B A	0.0003333	MPER183
	B	0.001433	MBRA12	B	0.000067	MBRA12	B	3.333E-05	CM2177-2	B	0.0002	MBRA12
DAY 6	A	0.011767	MNGA2	A	0.0126	CM2177-2	A	0.0003	MNGA2	A	0.0013333	MCOL22
	B A	0.008067	CM2177-2	B A	0.002067	MNGA2	A	0.0002667	MPER183	A	0.0012333	MNGA2
	B A	0.007233	MPER183	B A	0.001367	MPER183	A	0.0002667	MCOL22	A	0.0004667	MBRA12
	B	0.0048	MBRA12	B A	0.001	MCOL22	A	0.0001667	MBRA12	A	0.0004	CM2177-2
	B	0.004033	MCOL22	B	0.000467	MVEN77	A	0.0001333	MVEN77	A	0.0003667	MPER183
	B	0.0037	MVEN77	B	0.000167	MBRA12	A	6.667E-05	CM2177-2	A	0.0003333	MVEN77
DAY 7	A	0.016267	CM2177-2	A	0.0185	CM2177-2	A	0.0003667	MPER183	A	0.0014	MNGA2
	A	0.012533	MNGA2	B	0.0043	MNGA2	B A	0.0002	MNGA2	B A	0.0009333	MCOL22
	A	0.006667	MVEN77	B	0.0031	MCOL22	B	0.0001333	CM2177-2	B A	0.0006	MBRA12
	A	0.006467	MCOL22	B	0.002067	MPER183	B	0.0001333	MCOL22	B A	0.0005	MVEN77
	A	0.0054	MPER183	B	0.001	MVEN77	B	0.0001	MBRA12	B A	0.0004	CM2177-2
	A	0.004933	MBRA12	B	0.000133	MBRA12	B	6.667E-05	MVEN77	B	0.0002	MPER183
Ln CURVE AREA	A	0.048917	CM2177-2	A	0.056567	CM2177-2	A	0.0024968	MNGA2	A	0.008958	MNGA2
	B	0.038914	MNGA2	B	0.011737	MNGA2	B A	0.0015154	MPER183	B	0.003941	MCOL22
	C	0.027423	MPER183	B	0.010345	MPER183	B A	0.0012159	MVEN77	B	0.003095	CM2177-2
	D	0.017079	MCOL22	C B	0.005568	MCOL22	B A	0.001099	CM2177-2	B	0.00263	MVEN77
	D	0.016166	MVEN77	C B	0.002813	MVEN77	B A	0.0010658	MCOL22	B	0.002247	MPER183
	D	0.012239	MBRA12	C	0.0005	MBRA12	B	0.0005498	MBRA12	B	0.002164	MBRA12

8.3.2.2 REGWQ grouping by cultivar for June 1999 samples group

	GLUC			CHIT			SCOPOLETIN			SCOPOLIN		
	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR
DAY 0	A	0.7106	MCOL22	A	3.028	MNGA2	A	12	MVEN77	A	2.75	MVEN77
	A	0.4743	MBRA12	A	2.828	CM2177-2	B A	4.088	MNGA2	A	2.385	MCOL22
	A	0.4154	CM2177-2	A	0.677	MPER183	B A	3.938	MPER183	A	2.086	MPER183
	A	0.2183	MNGA2	A	0.5	MVEN77	B	3.394	CM2177-2	A	1.189	MBRA12
	A	0.1876	MVEN77	A	0.009	MBRA12	B	2.738	MCOL22	A	1.171	MNGA2
	A	0.0486	MPER183	A	0	MCOL22	B	2.694	MBRA12	A	0.474	CM2177-2
DAY 1	A	0.4963	MNGA2	A	4.569	CM2177-2	A	22.016	MBRA12	A	9.268	MBRA12
	A	0.4694	MCOL22	A	3.274	MNGA2	A	18.526	MVEN77	A	8.497	CM2177-2
	A	0.3706	CM2177-2	A	0.729	MPER183	B A	13.405	CM2177-2	A	7.39	MPER183
	A	0.2483	MBRA12	A	0.432	MVEN77	B A	10.449	MPER183	A	6.518	MCOL22
	A	0.2077	MVEN77	A	0.309	MCOL22	B	4.206	MCOL22	A	4.771	MVEN77
	A	0.0685	MPER183	A	0.164	MBRA12	B	3.261	MNGA2	A	2.987	MNGA2
DAY 2	A	0.9096	MNGA2	A	3.967	MNGA2	A	38.495	MCOL22	A	21.979	CM2177-2
	A	0.7975	CM2177-2	A	2.137	CM2177-2	A	35.747	MVEN77	A	17.18	MCOL22
	A	0.2979	MCOL22	A	0.721	MVEN77	B A	25.498	CM2177-2	A	12.847	MBRA12
	A	0.2159	MBRA12	A	0.655	MPER183	B A	24.604	MBRA12	A	11.851	MVEN77
	A	0.1488	MPER183	A	0.654	MCOL22	B A	17.204	MPER183	A	11.388	MPER183
	A	0.1193	MVEN77	A	0.273	MBRA12	B	4.541	MNGA2	A	4.594	MNGA2
DAY 3	A	1.3365	CM2177-2	A	2.3948	MNGA2	A	36.189	MVEN77	A	58.99	CM2177-2
	A	1.0889	MNGA2	A	1.9579	CM2177-2	A	29.42	CM2177-2	B A	37.48	MVEN77
	A	0.663	MCOL22	A	1.365	MCOL22	A	26.018	MPER183	B A	35.7	MCOL22
	A	0.412	MBRA12	A	0.7298	MPER183	A	21.057	MCOL22	B	16.17	MPER183
	A	0.3573	MPER183	A	0.4279	MVEN77	A	11.993	MBRA12	B	15.95	MBRA12
	A	0.2865	MVEN77	A	0.3646	MBRA12	A	5.945	MNGA2	B	8.67	MNGA2
DAY 4	A	2.989	MNGA2	A	1.9439	CM2177-2	A	38.133	CM2177-2	A	48.92	CM2177-2
	A	2.392	MCOL22	B	0.8354	MNGA2	A	27.962	MVEN77	A	28.17	MNGA2
	A	2.115	CM2177-2	B	0.8282	MCOL22	A	22.068	MPER183	A	27.54	MVEN77
	A	1.337	MVEN77	B	0.6147	MBRA12	A	16.488	MNGA2	A	24.66	MCOL22
	A	1.212	MBRA12	B	0.546	MPER183	A	15.207	MCOL22	A	16.7	MPER183
	A	0.641	MPER183	B	0.4555	MVEN77	A	13.437	MBRA12	A	16.29	MBRA12
DAY 5	A	3.808	CM2177-2	A	9.492	CM2177-2	A	61.1	CM2177-2	A	57.774	CM2177-2
	A	2.974	MCOL22	B	1.137	MCOL22	B A	28.32	MVEN77	B A	44.774	MNGA2
	A	2.852	MVEN77	B	1.004	MNGA2	B A	20.9	MNGA2	B C	27.037	MCOL22
	A	1.573	MNGA2	B	0.791	MPER183	B	14.81	MCOL22	B C	19.938	MVEN77
	A	1.192	MBRA12	B	0.528	MVEN77	B	13.76	MPER183	B C	13.364	MBRA12
	A	1.184	MPER183	B	0.459	MBRA12	B	8.13	MBRA12	C	8.933	MPER183
DAY 6	A	2.8186	CM2177-2	A	2.7859	CM2177-2	A	40.076	MVEN77	A	46	CM2177-2
	A	2.7056	MCOL22	B	0.8848	MNGA2	B A	24.856	MCOL22	A	42.23	MVEN77
	A	2.7005	MBRA12	B	0.772	MCOL22	B A	21.76	CM2177-2	A	39.04	MBRA12
	A	2.044	MNGA2	B	0.5408	MPER183	B	13.883	MPER183	A	33.09	MCOL22
	A	1.7972	MVEN77	B	0.4677	MBRA12	B	10.605	MNGA2	A	17.85	MNGA2
	A	0.944	MPER183	B	0.451	MVEN77	B	10.102	MBRA12	A	11.82	MPER183
DAY 7	A	8.367	CM2177-2	A	9.274	CM2177-2	A	29.471	CM2177-2	A	32.5	MVEN77
	B	3.27	MBRA12	B A	1.537	MCOL22	A	20.883	MVEN77	A	27.57	CM2177-2
	B	2.614	MNGA2	B A	1.389	MNGA2	A	16.053	MBRA12	A	22.62	MCOL22
	B	2.556	MCOL22	B	0.709	MPER183	A	16.046	MPER183	A	20.14	MPER183
	B	2.274	MVEN77	B	0.575	MVEN77	A	11.131	MCOL22	A	17.7	MNGA2
	B	0.689	MPER183	B	0.253	MBRA12	A	2.958	MNGA2	A	11.51	MBRA12
Ln CURVE AREA	A	2.8025	CM2177-2	A	3.3311	CM2177-2	A	5.3178	CM2177-2	A	5.5466	CM2177-2
	B	2.3363	MCOL22	B A	2.4584	MNGA2	A	5.3105	MVEN77	B A	5.0873	MVEN77
	B	2.2926	MNGA2	B	1.8742	MCOL22	B A	4.8292	MCOL22	B A	5.0264	MCOL22
	B	2.1507	MBRA12	B	1.7377	MPER183	B	4.7327	MPER183	B A	4.716	MNGA2
	B	2.0788	MVEN77	B	1.4524	MVEN77	B C	4.5946	MBRA12	B	4.6424	MBRA12
	C	1.5465	MPER183	B	1.2105	MBRA12	C	4.177	MNGA2	B	4.4205	MPER183

8.3.2.2 REGWQ grouping by cultivar for June 1999 samples group

	PPD-MARKER			PHEN-CON			TANNIN		
	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR	GROUP	MEAN	CULTIVAR
DAY 0	A	0	CM2177-2	A	48.596	MCOL22	A	2554	MCOL22
	B	0	MBRA12	B A	39.546	MVEN77	B A	1040.6	MNGA2
	C	0	MCOL22	B C	22.596	MPER183	B A	1007.6	MVEN77
	D	0	MNGA2	B C	20.033	MBRA12	B A	915.9	MBRA12
	E	0	MPER183	C	15.684	CM2177-2	B	718.9	MPER183
	F	0	MVEN77	C	14.576	MNGA2	B	518.5	CM2177-2
DAY 1	A	0.1421	MPER183	A	38.128	MCOL22	A	2382.1	MCOL22
	A	0	MBRA12	B A	36.305	MVEN77	A	1811.4	MNGA2
	A	0	MCOL22	C B A	20.275	MBRA12	A	954	MVEN77
	A	0	MNGA2	C B A	19.925	MPER183	A	758.2	MPER183
	A	0	CM2177-2	C B	14.524	CM2177-2	A	689.9	CM2177-2
	A	0	MVEN77	C	11.545	MNGA2	A	567	MBRA12
DAY 2	A	0.4025	MPER183	A	37.923	MCOL22	A	1267.7	MCOL22
	B	0.13653	MCOL22	A	33.738	MVEN77	A	1097.7	MNGA2
	B	0.07723	MVEN77	B	18.401	MBRA12	A	858	MVEN77
	B	0.07597	MBRA12	B	14.959	MPER183	A	637.1	MPER183
	B	0.03287	MNGA2	B	11.569	MNGA2	A	636.6	MBRA12
	B	0.00803	CM2177-2	B	9.655	CM2177-2	A	591.8	CM2177-2
DAY 3	A	3.079	MVEN77	A	40.825	MCOL22	A	1657.9	MCOL22
	A	0.972	MPER183	B A	31.117	MVEN77	B	793.8	MVEN77
	A	0.603	CM2177-2	B	20.205	MPER183	B	715	MPER183
	A	0.367	MCOL22	B	15.976	CM2177-2	B	606.6	MBRA12
	A	0.09	MBRA12	B	15.929	MBRA12	B	580.4	MNGA2
	A	0.058	MNGA2	B	11.799	MNGA2	B	543.5	CM2177-2
DAY 4	A	3.832	MVEN77	A	41.214	MCOL22	A	2603.5	MCOL22
	A	1.084	MCOL22	A	35.37	MVEN77	A	987.5	MVEN77
	A	0.466	MPER183	B	17.598	MBRA12	A	654	MBRA12
	A	0.417	CM2177-2	B	13.694	MPER183	A	642.1	MPER183
	A	0.367	MNGA2	B	13.515	CM2177-2	A	628.1	MNGA2
	A	0.29	MBRA12	B	12.558	MNGA2	A	484.9	CM2177-2
DAY 5	A	2.6433	CM2177-2	A	37.324	MCOL22	A	1991.1	MCOL22
	A	0.8862	MBRA12	B	27.324	MVEN77	B	1033.6	MVEN77
	A	0.5629	MVEN77	B	26.779	CM2177-2	B	992.7	CM2177-2
	A	0.4396	MPER183	C B	16.943	MBRA12	B	902.3	MBRA12
	A	0.3328	MNGA2	C	14.819	MPER183	B	538	MNGA2
	A	0.2505	MCOL22	C	14.003	MNGA2	B	535.5	MPER183
DAY 6	A	2.0071	MVEN77	A	41.134	MVEN77	A	1372.8	MCOL22
	A	1.9241	MPER183	B A	31.371	MCOL22	A	947.5	CM2177-2
	A	1.6099	MCOL22	B A	24.206	CM2177-2	A	842.9	MVEN77
	A	0.6738	CM2177-2	B	13.514	MPER183	A	759.6	MPER183
	A	0.5396	MBRA12	B	12.553	MBRA12	A	668.7	MBRA12
	A	0.3358	MNGA2	B	12.477	MNGA2	A	658.9	MNGA2
DAY 7	A	2.186	CM2177-2	A	34.972	MCOL22	A	2479	MCOL22
	A	1.95	MBRA12	B A	27.523	MVEN77	B	1337.1	CM2177-2
	A	1.19	MPER183	B A	23.518	CM2177-2	B	856.4	MVEN77
	A	0.945	MVEN77	B C	16.714	MPER183	B	783.2	MPER183
	A	0.495	MCOL22	B C	13.89	MBRA12	B	711.1	MBRA12
	A	0.089	MNGA2	C	6.447	MNGA2	B	687.7	MNGA2
Ln CURVE AREA	A	2.1502	MVEN77	A	5.58848	MCOL22	A	9.52807	MCOL22
	A	1.8142	CM2177-2	A	5.4768	MVEN77	B	8.76441	MVEN77
	A	1.7329	MPER183	B	4.82994	CM2177-2	C B	8.71818	MNGA2
	A	1.4981	MCOL22	B	4.78025	MBRA12	C B	8.55157	CM2177-2
	A	1.3415	MBRA12	B	4.76618	MPER183	C	8.47938	MBRA12
	A	0.7138	MNGA2	C	4.44412	MNGA2	C	8.47575	MPER183

8.3.2.3 REGWQ grouping by cultivar for December 1999 samples group

	POX			SCP-POX			PPO		
	GRP	MEAN	CULTIVAR	GR	MEAN	CULTIVAR	GRP	MEAN	CULTIVAR
DAY 0	A	0.0023671	b_MPER183_0	A	0.014508	m_MCOL22_0	A	0.00020157	b_MBRA337_0
	B A	0.0015695	a_CM7033-3_0	A	0.012068	b_MBRA337_0	A	0.0001231	m_MNGA2_0
	B A	0.001493	a_SM985-9_0	A	0.007104	a_CM7033-3_0	A	0.00011803	m_MCOL22_0
	B A	0.0014289	b_MBRA337_0	A	0.004993	b_MPER183_0	A	0.00011377	m_MVEN77_0
	B	0.001341	m_MNGA2_0	A	0.004139	a_SM985-9_0	A	0.0000839	a_SM985-9_0
	B	0.0012012	a_MDOM5_0	A	0.003845	m_MNGA2_0	A	0.00007307	b_MPER183_0
	B	0.0011952	a_CM2177-2_0	A	0.003625	a_CM2177-2_0	A	0.0000382	b_MBRA12_0
	B	0.0011839	m_MVEN77_0	A	0.003344	m_MVEN77_0	A	0.0000314	a_MDOM5_0
	B	0.000906	b_MBRA12_0	A	0.003013	b_MBRA12_0	A	0.0000295	a_CM2177-2_0
	B	0.0008871	m_MCOL22_0	A	0.003004	a_MDOM5_0	A	0.00002227	a_CM7033-3_0
DAY 1	A	0.0035338	b_MPER183_1	A	0.027164	m_MCOL22_1	A	0.0020178	b_MPER183_1
	B	0.0024767	m_MNGA2_1	A	0.010819	a_CM7033-3_1	A	0.0018936	a_CM2177-2_1
	B	0.002009	a_CM2177-2_1	A	0.007588	a_SM985-9_1	A	0.0016239	a_SM985-9_1
	B	0.001918	a_SM985-9_1	A	0.007253	b_MBRA337_1	B	0.0005434	b_MBRA337_1
	B	0.0018415	m_MVEN77_1	A	0.006346	a_CM2177-2_1	B	0.0004391	a_MDOM5_1
	C B	0.0017138	a_CM7033-3_1	A	0.005065	a_MDOM5_1	B	0.0001365	m_MVEN77_1
	C B	0.0016924	a_MDOM5_1	A	0.004785	m_MNGA2_1	B	0.000076	m_MNGA2_1
	C B	0.0016403	m_MCOL22_1	A	0.004541	b_MPER183_1	B	0.00004	m_MCOL22_1
	C B	0.0016284	b_MBRA337_1	A	0.004057	m_MVEN77_1	B	0.0000353	b_MBRA12_1
	C	0.0008997	b_MBRA12_1	A	0.003194	b_MBRA12_1	B	0.0000327	a_CM7033-3_1
DAY 2	A	0.0034749	b_MPER183_2	A	0.026042	m_MCOL22_2	A	0.0028125	b_MPER183_2
	A	0.0030661	a_SM985-9_2	A	0.02027	a_SM985-9_2	A	0.0024485	a_SM985-9_2
	A	0.0024477	b_MBRA337_2	A	0.014543	a_MDOM5_2	A	0.0022533	a_CM2177-2_2
	A	0.0020735	m_MNGA2_2	A	0.010765	a_CM2177-2_2	B A	0.0013777	a_MDOM5_2
	A	0.0020622	a_MDOM5_2	A	0.010712	a_CM7033-3_2	B A	0.0013574	b_MBRA337_2
	A	0.0018552	m_MVEN77_2	A	0.00947	b_MBRA337_2	B	0.0003933	a_CM7033-3_2
	A	0.0016795	a_CM7033-3_2	A	0.009277	m_MVEN77_2	B	0.0003586	m_MCOL22_2
	A	0.0014703	a_CM2177-2_2	A	0.007329	b_MPER183_2	B	0.0003113	m_MNGA2_2
	A	0.0014301	m_MCOL22_2	A	0.007277	m_MNGA2_2	B	0.0002569	m_MVEN77_2
	A	0.0012561	b_MBRA12_2	A	0.005138	b_MBRA12_2	B	0.0000733	b_MBRA12_2
DAY 3	A	0.0031952	b_MPER183_3	A	0.028333	a_MDOM5_3	A	0.0033284	b_MPER183_3
	B A	0.0022761	a_MDOM5_3	A	0.025702	a_SM985-9_3	B A	0.0026348	a_CM2177-2_3
	C B A	0.0019143	m_MNGA2_3	A	0.020797	a_CM2177-2_3	B A	0.0025238	a_SM985-9_3
	C B	0.0016598	b_MBRA337_3	A	0.019234	m_MVEN77_3	B A	0.0022002	b_MBRA337_3
	C B	0.0015805	a_SM985-9_3	A	0.014303	b_MPER183_3	B A	0.0016897	a_MDOM5_3
	C B	0.0015047	a_CM7033-3_3	A	0.014272	a_CM7033-3_3	B A	0.0007123	m_MCOL22_3
	C B	0.0014812	m_MVEN77_3	A	0.013921	m_MNGA2_3	B	0.0005112	a_CM7033-3_3
	C B	0.0008039	a_CM2177-2_3	A	0.013728	b_MBRA337_3	B	0.0003387	m_MVEN77_3
	C B	0.000711	m_MCOL22_3	A	0.009312	b_MBRA12_3	B	0.0003106	b_MBRA12_3
	C	0.0006205	b_MBRA12_3	A	0.007763	m_MCOL22_3	B	0.0002243	m_MNGA2_3
DAY 4	A	0.002575	b_MPER183_4	A	0.046276	m_MVEN77_4	A	0.0036893	b_MPER183_4
	B A	0.00223	a_MDOM5_4	B A	0.037696	a_SM985-9_4	B A	0.0027944	a_SM985-9_4
	C B A	0.0019949	m_MNGA2_4	B A	0.034186	a_CM2177-2_4	C B A	0.002425	a_CM2177-2_4
	D C B A	0.0017409	m_MVEN77_4	B A	0.032797	a_MDOM5_4	D C B A	0.002081	a_MDOM5_4
	D C B A	0.0016722	a_CM7033-3_4	B C	0.024573	b_MBRA337_4	D C B E	0.0012997	b_MBRA337_4
	E D C B A	0.0013685	a_SM985-9_4	B C	0.018473	b_MPER183_4	D C E	0.0009018	a_CM7033-3_4
	E D C B	0.0011826	b_MBRA337_4	C	0.013657	m_MNGA2_4	D C E	0.0008368	m_MVEN77_4
	E D C	0.0007463	a_CM2177-2_4	C	0.013109	a_CM7033-3_4	D E	0.0007149	m_MNGA2_4
	E D	0.0003974	b_MBRA12_4	C	0.009503	b_MBRA12_4	D E	0.0006095	m_MCOL22_4
	E	0.000278	m_MCOL22_4	C	0.006051	m_MCOL22_4	E	0.0002911	b_MBRA12_4
Ln CURVE AREA	A	0.012589	MPER183	A	0.07173	SM985-9	A	0.009989	MPER183
	B	0.008098	MNGA2	A	0.06761	MCOL22	B A	0.008003	SM985-9
	B	0.007963	SM985-9	A	0.06357	MDOM5	B A	0.007977	CM2177-2
	C B	0.007715	MDOM5	A	0.05579	MVEN77	B C	0.004834	MBRA337
	C B	0.007015	MBRA337	A	0.05502	CM2177-2	B C	0.004551	MDOM5
	C B	0.006618	MVEN77	A	0.04754	MBRA337	C	0.001473	MCOL22
	C B	0.006497	CM7033-3	A	0.04484	CM7033-3	C	0.001398	CM7033-3
	C B	0.005239	CM2177-2	A	0.03717	MPER183	C	0.001206	MVEN77
	C B	0.004354	MCOL22	A	0.03411	MNGA2	C	0.00103	MNGA2
	C	0.003422	MBRA12	A	0.02361	MBRA12	C	0.000584	MBRA12

8.3.2.3 REGWQ grouping by cultivar for December 1999 samples group

	CAT			SCOPOLETIN			SCOPOLIN		
	GRP	MEAN	CULTIVAR	GRP	MEAN	CULTIVAR	GRP	MEAN	CULTIVAR
DAY 0	A	0.0007085	b_MPER183_0	A	13.344	m_MNGA2_0	A	7.891	m_MCOL22_0
	A	0.000645	m_MNGA2_0	A	9.808	a_SM985-9_0	A	7.877	b_MBRA337_0
	A	0.0005228	a_CM2177-2_0	A	9.534	m_MVEN77_0	A	3.468	m_MNGA2_0
	A	0.0003959	a_SM985-9_0	A	9.154	a_MDOM5_0	A	2.268	m_MVEN77_0
	A	0.0003582	m_MVEN77_0	A	8.334	b_MBRA337_0	A	2.236	a_SM985-9_0
	A	0.000313	a_CM7033-3_0	A	7.636	m_MCOL22_0	A	1.898	a_CM2177-2_0
	A	0.0003078	a_MDOM5_0	A	6.279	a_CM2177-2_0	A	0.785	b_MPER183_0
	A	0.0002807	m_MCOL22_0	A	4.482	b_MPER183_0	A	0.767	a_MDOM5_0
	A	0.0002507	b_MBRA337_0	A	3.132	b_MBRA12_0	A	0.73	b_MBRA12_0
	A	0.0001911	b_MBRA12_0	A	3.008	a_CM7033-3_0	A	0.647	a_CM7033-3_0
DAY 1	A	0.0007593	m_MNGA2_1	A	50.15	a_MDOM5_1	A	49.715	a_SM985-9_1
	B A	0.0006229	a_SM985-9_1	A	49.23	a_CM7033-3_1	B	18.908	b_MBRA337_1
	B A	0.0005613	a_CM2177-2_1	B A	38.9	a_SM985-9_1	B	18.033	m_MNGA2_1
	B A	0.0004889	m_MCOL22_1	B A	24.98	m_MVEN77_1	B	14.021	m_MCOL22_1
	B A	0.0004827	b_MPER183_1	B A	20.95	b_MPER183_1	B	11.105	a_CM2177-2_1
	B A	0.0004388	a_MDOM5_1	B A	18.72	a_CM2177-2_1	B	10.196	a_MDOM5_1
	B A	0.0003997	b_MBRA337_1	B A	17.33	b_MBRA337_1	B	6.388	b_MPER183_1
	B A	0.0003631	a_CM7033-3_1	B A	17.01	m_MNGA2_1	B	5.639	a_CM7033-3_1
	B	0.0002727	m_MVEN77_1	B	12.31	b_MBRA12_1	B	5.353	b_MBRA12_1
	B	0.0002092	b_MBRA12_1	B	7.66	m_MCOL22_1	B	4.267	m_MVEN77_1
DAY 2	A	0.0007932	m_MNGA2_2	A	60.72	m_MVEN77_2	A	52.98	b_MBRA337_2
	A	0.0007808	a_MDOM5_2	A	46.16	a_MDOM5_2	B A	44.24	a_SM985-9_2
	B A	0.0007235	a_CM2177-2_2	A	45.88	a_CM2177-2_2	B A	35.11	a_CM2177-2_2
	B A	0.0006811	b_MPER183_2	A	37.02	a_CM7033-3_2	B A	27.72	m_MNGA2_2
	B A	0.00066	a_SM985-9_2	A	35.26	b_MPER183_2	B A	24.07	m_MCOL22_2
	B A	0.000642	b_MBRA337_2	A	26.84	b_MBRA337_2	B	16.41	a_MDOM5_2
	B A	0.0004584	a_CM7033-3_2	A	25.92	m_MNGA2_2	B	16.28	m_MVEN77_2
	B A	0.0004224	m_MVEN77_2	A	24.06	b_MBRA12_2	B	16.09	b_MPER183_2
	B	0.0003609	m_MCOL22_2	A	23.67	m_MCOL22_2	B	14.75	a_CM7033-3_2
	B	0.0003594	b_MBRA12_2	A	18.81	a_SM985-9_2	B	8.77	b_MBRA12_2
DAY 3	A	0.0007781	m_MNGA2_3	A	68.01	m_MVEN77_3	A	71.8	a_SM985-9_3
	A	0.000735	b_MPER183_3	B A	52.06	a_MDOM5_3	B A	64.86	a_CM2177-2_3
	A	0.0006997	b_MBRA337_3	B A	50.45	a_SM985-9_3	B A	48.81	a_MDOM5_3
	A	0.000661	a_CM2177-2_3	B A	40.58	b_MPER183_3	B A	38.83	m_MVEN77_3
	A	0.0005228	a_MDOM5_3	B A	37.13	b_MBRA12_3	B A	35.45	m_MCOL22_3
	A	0.0005023	a_SM985-9_3	B A	36.21	a_CM2177-2_3	B A	32.78	m_MNGA2_3
	A	0.0004987	a_CM7033-3_3	B A	30.56	m_MCOL22_3	B A	29.42	b_MBRA337_3
	A	0.0004656	m_MVEN77_3	B A	27.58	a_CM7033-3_3	B A	28.42	a_CM7033-3_3
	A	0.00035	m_MCOL22_3	B	17.54	m_MNGA2_3	B A	23.31	b_MPER183_3
	A	0.0002107	b_MBRA12_3	B	17.53	b_MBRA337_3	B	19.24	b_MBRA12_3
DAY 4	A	0.0009514	b_MPER183_4	A	80.71	m_MVEN77_4	A	91.41	a_SM985-9_4
	A	0.0008646	m_MNGA2_4	B A	50.75	b_MBRA12_4	B A	66.89	a_CM2177-2_4
	B A	0.000712	b_MBRA337_4	B	39.84	a_MDOM5_4	B C	39.94	b_MBRA337_4
	B A	0.0006314	a_CM2177-2_4	B	36.43	a_CM2177-2_4	B C	35.88	m_MNGA2_4
	B A	0.0005889	a_MDOM5_4	B	30.69	a_SM985-9_4	B C	33.91	b_MBRA12_4
	B A	0.0004483	m_MVEN77_4	B	27.16	b_MPER183_4	B C	31.18	a_MDOM5_4
	B A	0.0004321	a_CM7033-3_4	B	17.11	b_MBRA337_4	B C	30.95	m_MVEN77_4
	B	0.0002825	b_MBRA12_4	B	17.07	m_MCOL22_4	B C	23.26	m_MCOL22_4
	B	0.00024	a_SM985-9_4	B	16.59	a_CM7033-3_4	C	18.36	b_MPER183_4
	B	0.0002174	m_MCOL22_4	B	15.74	m_MNGA2_4	C	15.36	a_CM7033-3_4
Ln CURVE AREA	A	0.0030804	MNGA2	A	5.2843	MVEN77	A	5.35	SM985-9
	B A	0.0027249	MPER183	B A	5.1349	MDOM5	B A	4.9578	CM2177-2
	C B A	0.0025196	CM2177-2	C B A	4.8427	SM985-9	C B A	4.8295	MBRA337
	D C B A	0.0022202	MBRA337	C B A	4.8139	CM7033-3	D C B A	4.5787	MNGA2
	D C B A	0.0021883	MDOM5	C B A	4.7781	CM2177-2	D C B A	4.5004	MDOM5
	D C B A	0.0021009	SM985-9	C B A	4.7107	MPER183	D C B A	4.4908	MCOL22
	D C B	0.0016913	CM7033-3	C B A	4.5976	MBRA12	D C B	4.3069	MVEN77
	D C B	0.0015627	MVEN77	C B	4.3139	MNGA2	D C	4.0082	MPER183
	D C	0.0014479	MCOL22	C B	4.2868	MBRA337	D	3.947	CM7033-3
	D	0.0010156	MBRA12	C	4.2072	MCOL22	D	3.9236	MBRA12

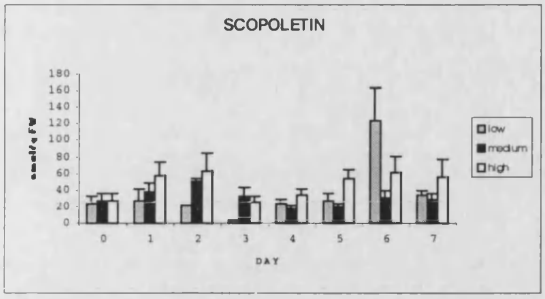
8.3.2.3 REGWQ grouping by cultivar for December 1999 samples group

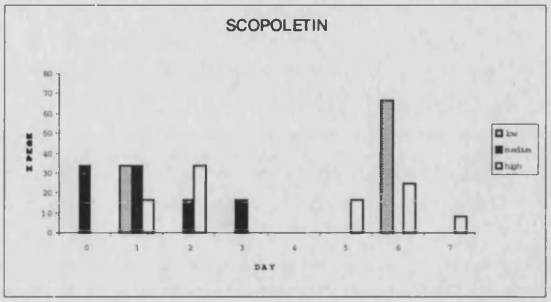
	ESCULIN			PPD-MARKER			PHEN-CON		
	GR	MEAN	CULTIVAR	GR	MEAN	CULTIVAR	GRP	MEAN	CULTIVAR
DAY 0	A	0.16397	a_CM2177-2_0	A	0	a_CM2177-2_0	A	52.415	b_MBRA337_0
	B	0	a_CM7033-3_0	B	0	a_CM7033-3_0	B A	41.982	a_MDOM5_0
	B	0	a_MDOM5_0	C	0	a_MDOM5_0	C B A	36.138	m_MCOL22_0
	B	0	a_SM985-9_0	D	0	a_SM985-9_0	C B A	29.445	m_MNGA2_0
	B	0	b_MBRA12_0	E	0	b_MBRA12_0	C B A	29.021	a_CM7033-3_0
	B	0	b_MBRA337_0	F	0	b_MBRA337_0	C B	21.958	a_CM2177-2_0
	B	0	b_MPER183_0	G	0	b_MPER183_0	C B	20.686	m_MVEN77_0
	B	0	m_MCOL22_0	H	0	m_MCOL22_0	C B	20.348	b_MBRA12_0
	B	0	m_MNGA2_0	I	0	m_MNGA2_0	C B	18.638	a_SM985-9_0
	B	0	m_MVEN77_0	J	0	m_MVEN77_0	C	12.938	b_MPER183_0
DAY 1	A	1.1007	a_MDOM5_1	A	0.20245	a_MDOM5_1	A	50.275	b_MBRA337_1
	A	0.6241	b_MBRA337_1	A	0.08976	a_SM985-9_1	B A	37.187	m_MCOL22_1
	A	0.3666	a_CM2177-2_1	A	0.06294	a_CM2177-2_1	B A	35.35	m_MVEN77_1
	A	0.236	a_SM985-9_1	A	0.06041	m_MVEN77_1	B A	28.074	a_MDOM5_1
	A	0.1442	m_MCOL22_1	A	0.04746	a_CM7033-3_1	B A	26.608	m_MNGA2_1
	A	0.1186	b_MPER183_1	A	0.03619	m_MNGA2_1	B A	26.291	a_CM7033-3_1
	A	0.1137	b_MBRA12_1	A	0.03069	b_MBRA337_1	B	20.967	b_MBRA12_1
	A	0.1003	m_MNGA2_1	A	0.01646	b_MPER183_1	B	20.894	a_SM985-9_1
	A	0.0546	a_CM7033-3_1	A	0.00524	b_MBRA12_1	B	18.539	a_CM2177-2_1
	A	0	m_MVEN77_1	A	0	m_MCOL22_1	B	16.729	b_MPER183_1
DAY 2	A	5.516	a_SM985-9_2	A	1.6398	a_SM985-9_2	A	49.042	m_MCOL22_2
	B A	2.771	a_MDOM5_2	B A	0.8192	a_MDOM5_2	B A	38.026	b_MBRA337_2
	B A	1.601	b_MBRA337_2	B A	0.586	m_MCOL22_2	B A	30.534	a_MDOM5_2
	B	1.278	a_CM2177-2_2	B A	0.5554	a_CM2177-2_2	B A	30.455	m_MVEN77_2
	B	0.695	a_CM7033-3_2	B A	0.4257	a_CM7033-3_2	B	27.338	m_MNGA2_2
	B	0.695	b_MPER183_2	B A	0.2784	m_MNGA2_2	B	26.046	b_MBRA12_2
	B	0.415	m_MCOL22_2	B A	0.2478	m_MVEN77_2	B	25.919	a_CM7033-3_2
	B	0.293	m_MNGA2_2	B	0.1332	b_MPER183_2	B	22.619	a_CM2177-2_2
	B	0.112	m_MVEN77_2	B	0.0768	b_MBRA12_2	B	19.079	b_MPER183_2
	B	0.082	b_MBRA12_2	B	0.0368	b_MBRA337_2	B	17.738	a_SM985-9_2
DAY 3	A	12.092	a_MDOM5_3	A	3.1117	a_MDOM5_3	A	40.334	b_MBRA337_3
	B A	4.053	a_SM985-9_3	A	2.1424	a_CM2177-2_3	A	30.963	b_MPER183_3
	B A	3.766	a_CM2177-2_3	A	1.8258	a_CM7033-3_3	A	30.93	m_MCOL22_3
	B A	2.658	a_CM7033-3_3	A	1.527	a_SM985-9_3	A	30.578	a_SM985-9_3
	B A	1.847	m_MVEN77_3	A	1.3451	m_MVEN77_3	A	29.667	a_CM7033-3_3
	B A	1.717	m_MCOL22_3	A	0.9314	b_MPER183_3	A	28.975	m_MVEN77_3
	B	1.123	b_MBRA337_3	A	0.6892	b_MBRA337_3	A	27.283	a_MDOM5_3
	B	1.106	b_MPER183_3	A	0.5938	m_MNGA2_3	A	23.369	m_MNGA2_3
	B	0.54	b_MBRA12_3	A	0.3243	m_MCOL22_3	A	20.387	b_MBRA12_3
	B	0.471	m_MNGA2_3	A	0.3073	b_MBRA12_3	A	18.072	a_CM2177-2_3
DAY 4	A	7.93	m_MVEN77_4	A	3.967	m_MVEN77_4	A	46.093	b_MBRA337_4
	A	7.32	a_MDOM5_4	A	2.721	a_SM985-9_4	A	45.449	m_MVEN77_4
	A	3.383	a_CM2177-2_4	A	2.459	a_MDOM5_4	B A	34.457	b_MBRA12_4
	A	2.29	a_SM985-9_4	A	1.873	b_MPER183_4	B A	32.655	m_MCOL22_4
	A	1.894	b_MPER183_4	A	1.786	a_CM2177-2_4	B A	29.675	a_MDOM5_4
	A	1.174	b_MBRA337_4	A	1.549	a_CM7033-3_4	B A	28.28	a_CM7033-3_4
	A	1.087	a_CM7033-3_4	A	1.506	m_MNGA2_4	B	27.135	a_SM985-9_4
	A	0.844	m_MNGA2_4	A	0.838	b_MBRA12_4	B	25.552	m_MNGA2_4
	A	0.83	b_MBRA12_4	A	0.376	m_MCOL22_4	B	21.408	a_CM2177-2_4
	A	0.083	m_MCOL22_4	A	0.35	b_MBRA337_4	B	18.882	b_MPER183_4
Ln CURVE AREA	A	2.8948	MDOM5	A	1.8449	MDOM5	A	5.1831	MBRA337
	A	2.1939	SM985-9	B A	1.6352	SM985-9	B A	5.0269	MCOL22
	A	1.8483	CM2177-2	B A	1.5225	MVEN77	C B A	4.848	MVEN77
	A	1.5774	MBRA337	B A	1.3661	CM2177-2	C B A	4.7954	MDOM5
	A	1.5505	MVEN77	B A	1.2372	CM7033-3	C B	4.7131	CM7033-3
	A	1.4623	CM7033-3	B A	0.9144	MPER183	C B	4.582	MNGA2
	A	1.2613	MPER183	B A	0.8538	MNGA2	C B	4.5583	MBRA12
	A	1.0206	MCOL22	B A	0.6709	MCOL22	C	4.5319	SM985-9
	A	0.7955	MNGA2	B	0.6134	MBRA337	C	4.4178	MPER183
	A	0.7253	MBRA12	B	0.5554	MBRA12	C	4.3987	CM2177-2

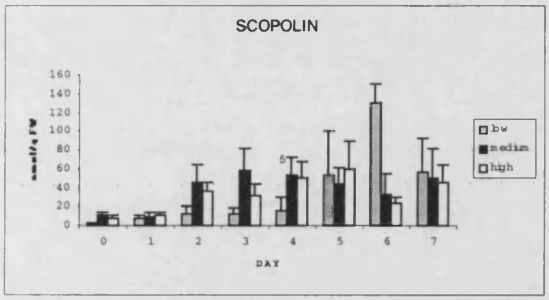
8.4 GRAPHS FOR AVERAGE AND PEAK FREQUENCY OF SECONDARY METABOLITES ACCUMULATION AND ENZYMATIC ACTIVITY

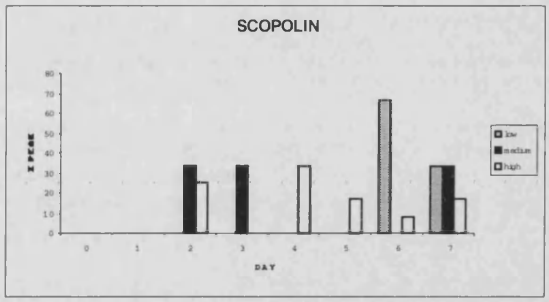
In order to easy visualise the general trends in secondary metabolites accumulation and enzymatic activity in the different groups of samples (Bath, December 1999, June 1999 and Family K), the average of the cultivars response was calculated dividing the samples in three groups based on the susceptibility of the cultivars towards PPD (low, medium and high). Additionally, the day of maximum accumulation of metabolites and enzyme activity between the three susceptibilities to PPD was determined. It was carried out by the calculation of the peak frequency, it means, the day of the PPD time course at which the highest value was observed. In this case, the quantifications of the three repetitions were not averaged, because the average in the frequency calculation is not statistically representative. This test will be referring as "frequency peak" in the manuscript.

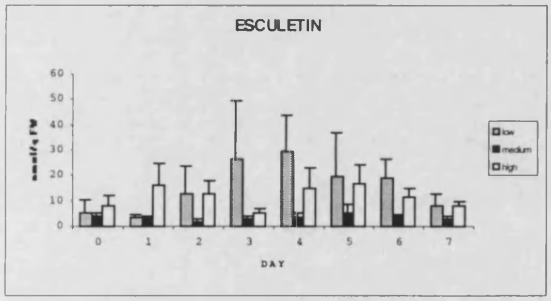
8.4.1 Graphs for average and peak frequency of secondary metabolites accumulation in Bath samples group

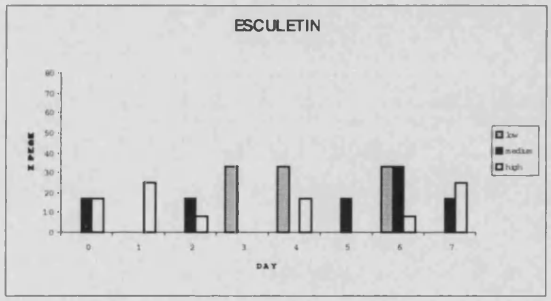


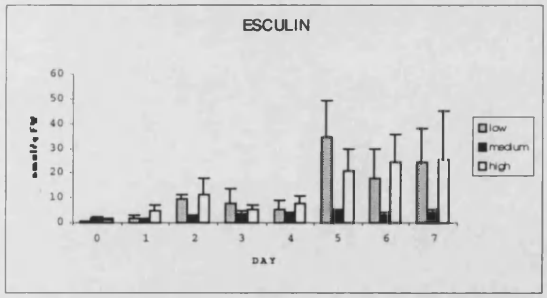


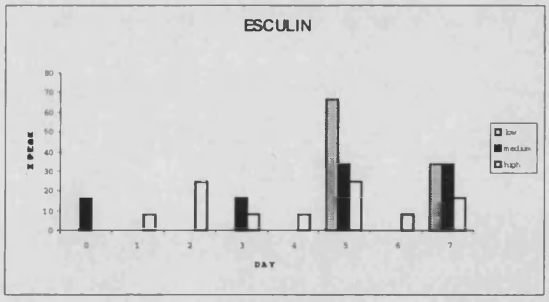




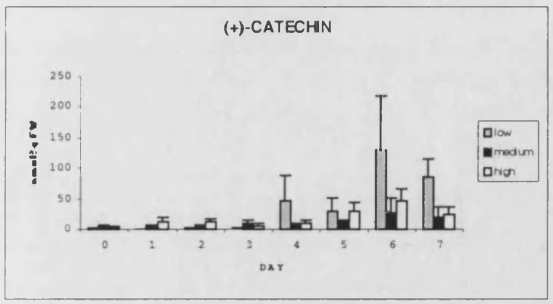


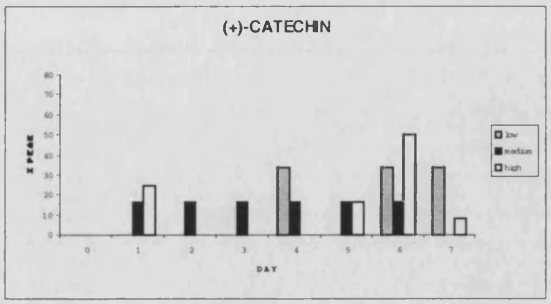


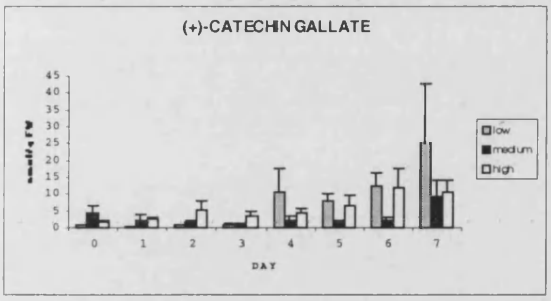


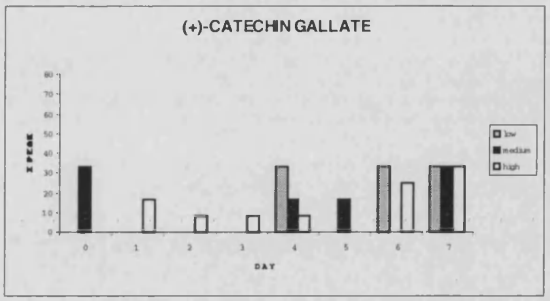


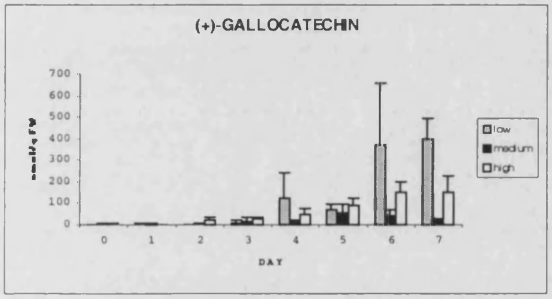
8.4.1 Graphs for average and peak frequency of secondary metabolites accumulation in Bath samples group

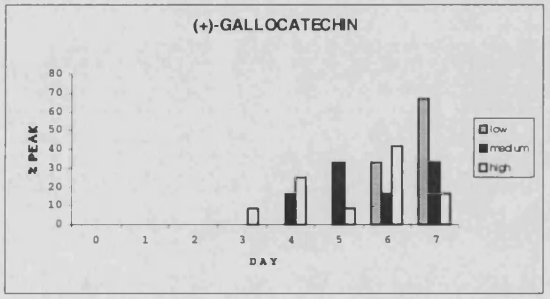




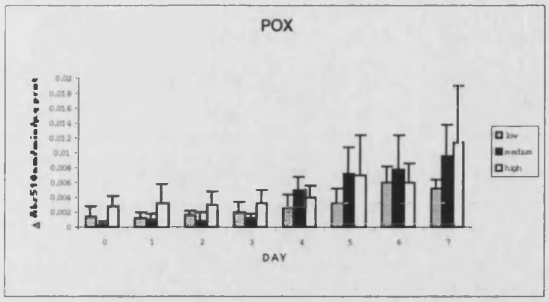


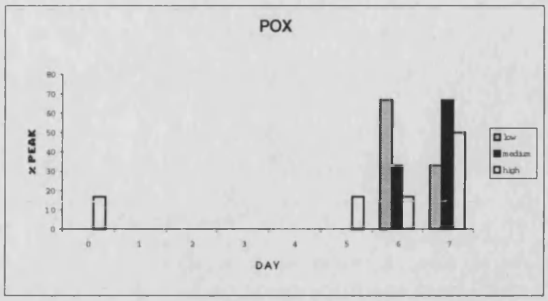


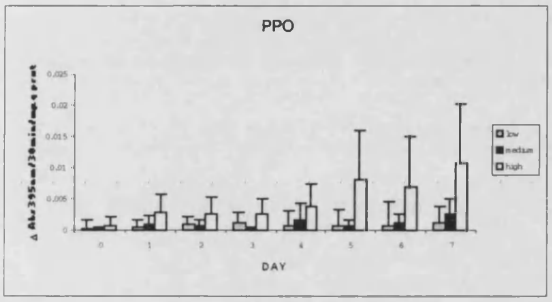


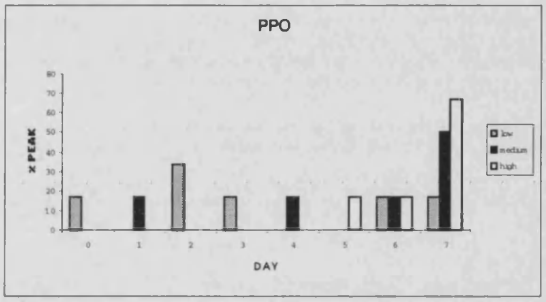


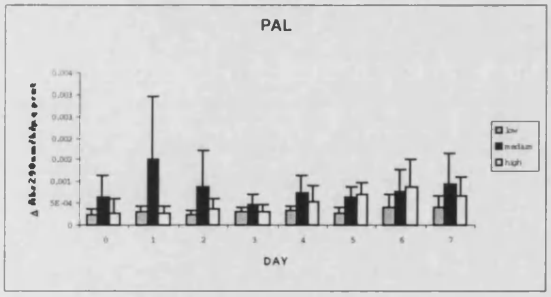
8.4.2 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in June 1999 samples group

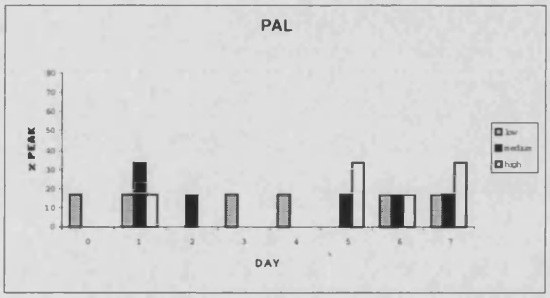


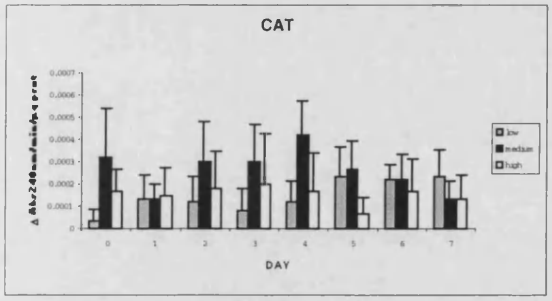


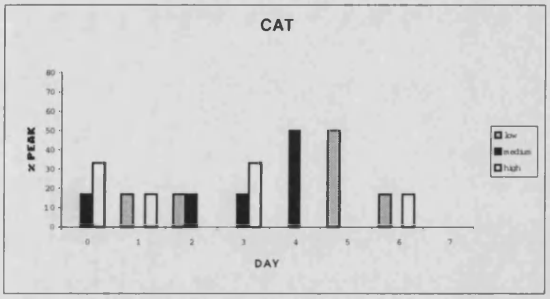




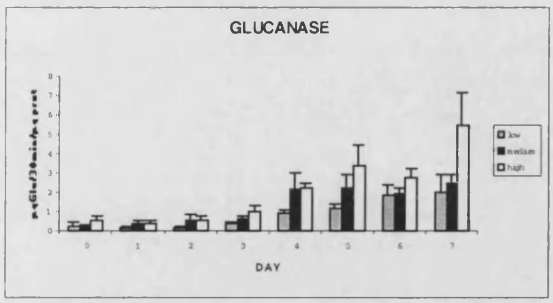


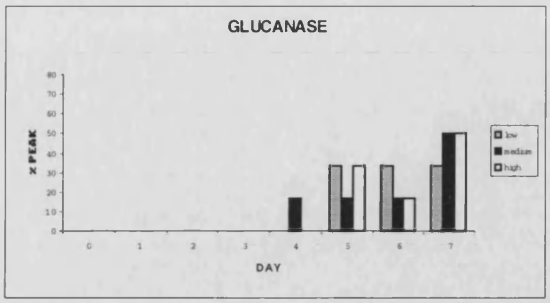


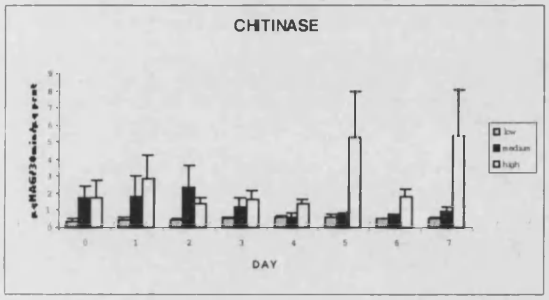


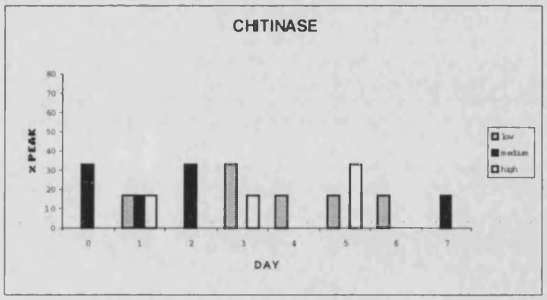


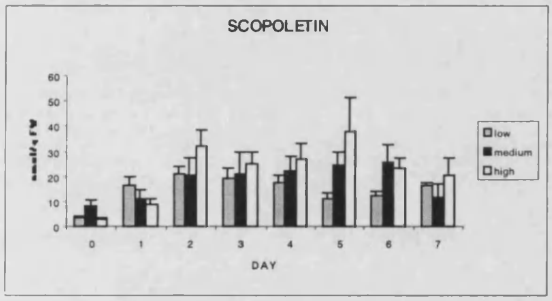
8.4.2 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in June 1999 samples group

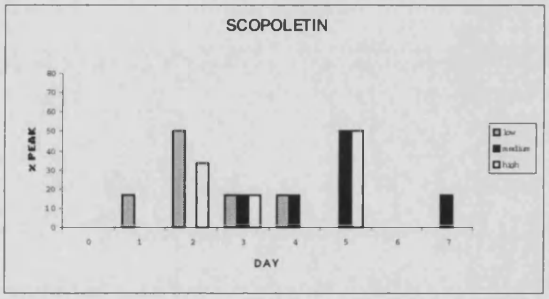


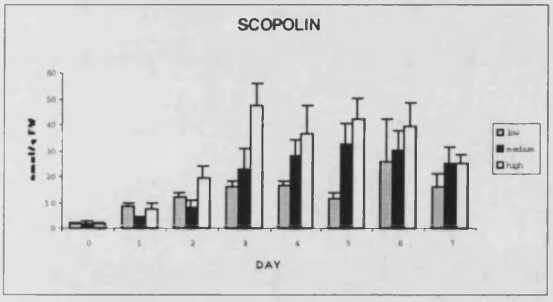


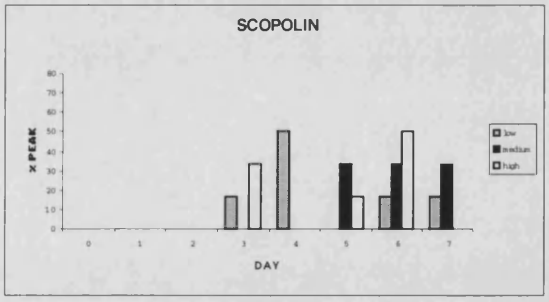




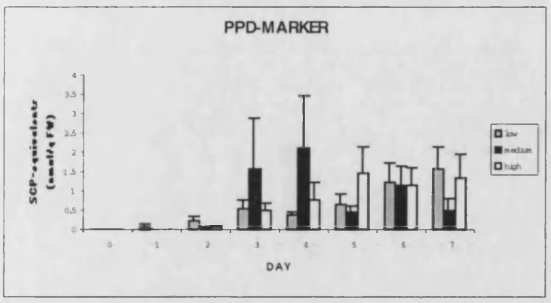


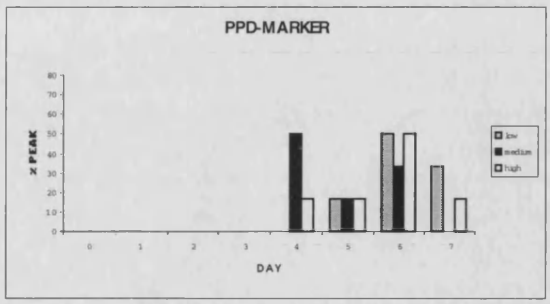


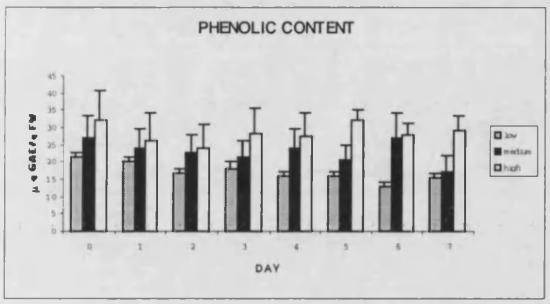


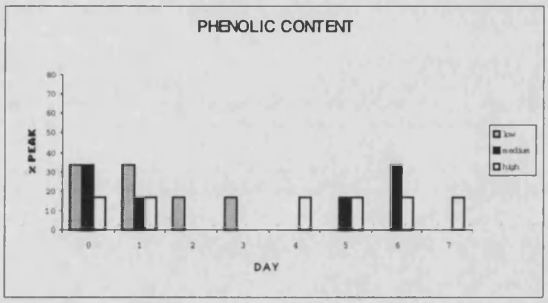


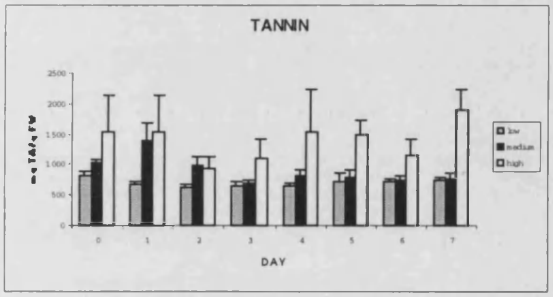
8.4.2 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in June 1999 samples group

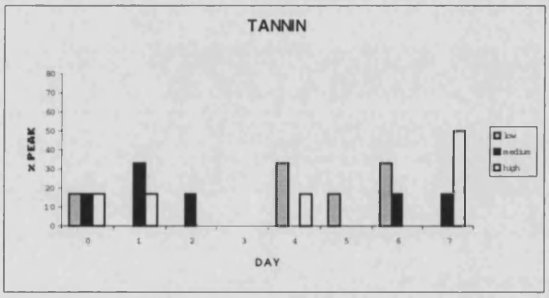




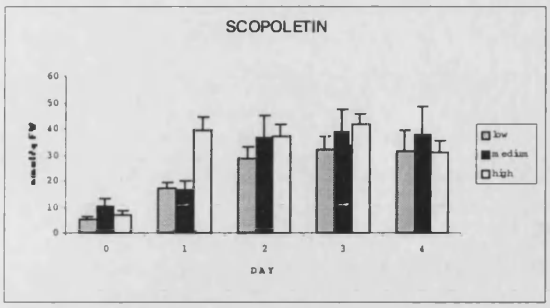


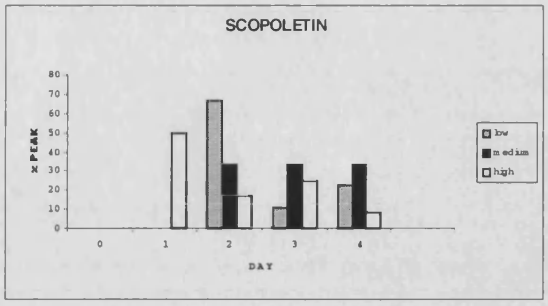


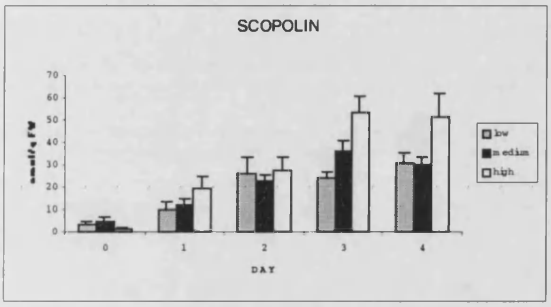


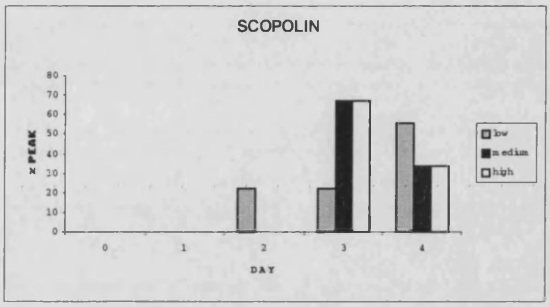


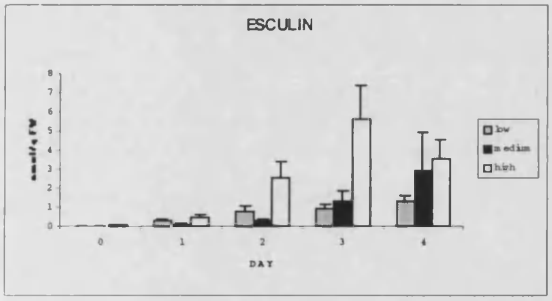
8.4.3 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in December 1999 samples group

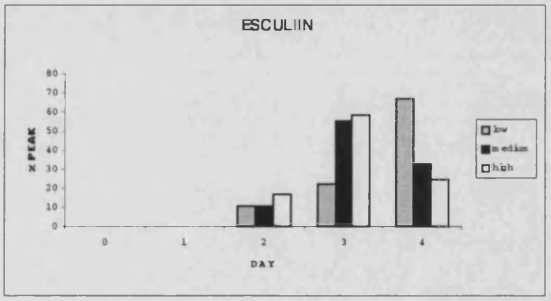


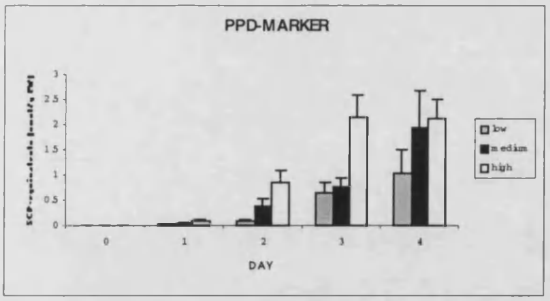


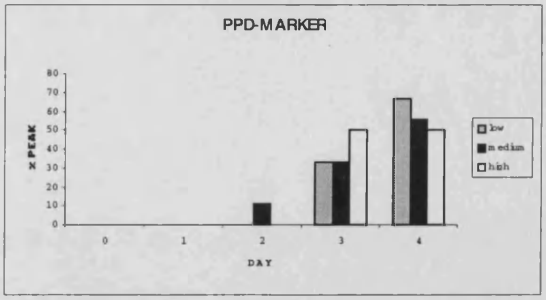


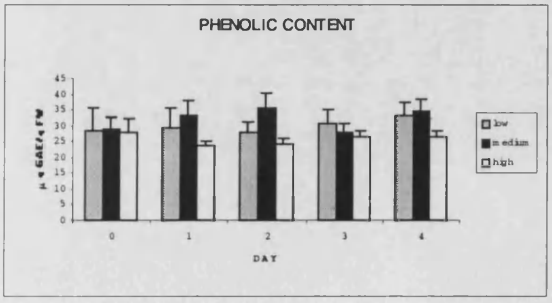


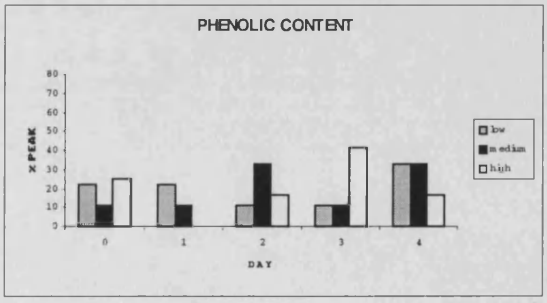




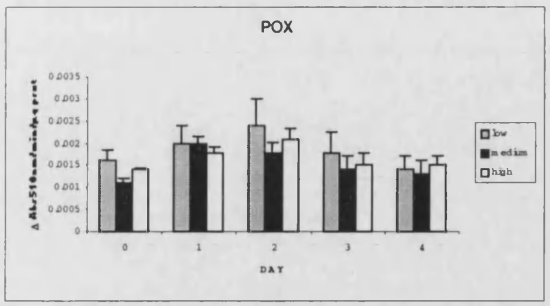


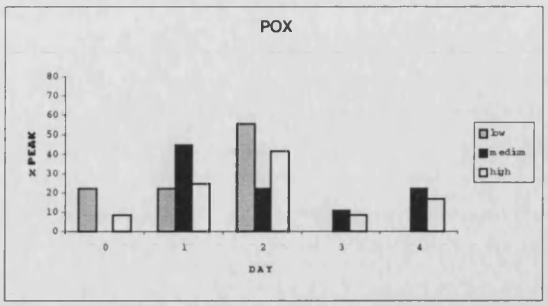


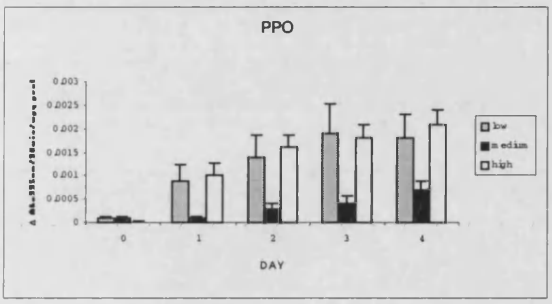


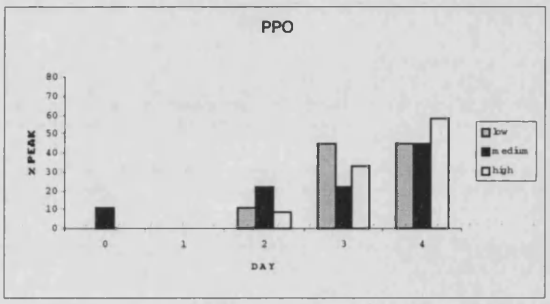


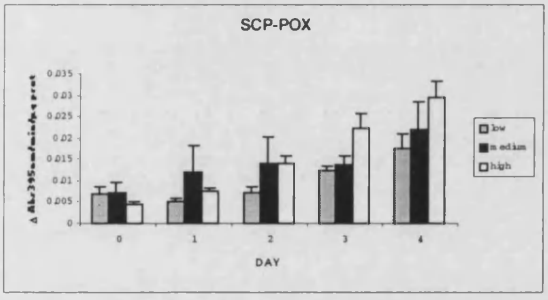
8.4.3 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in December 1999 samples group

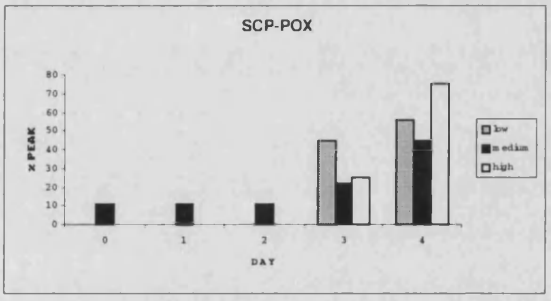


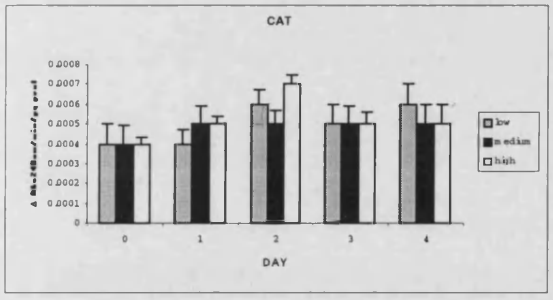


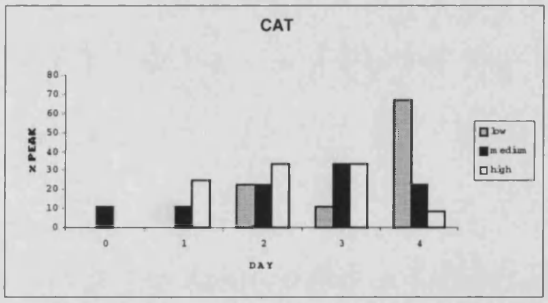




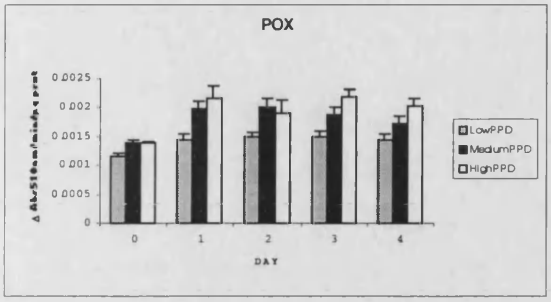


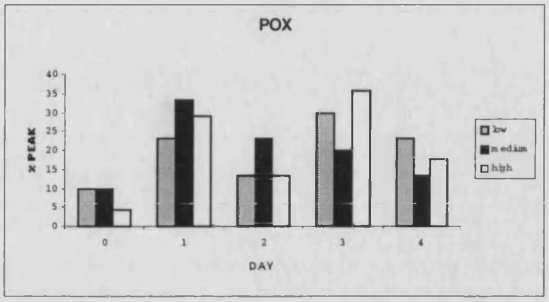


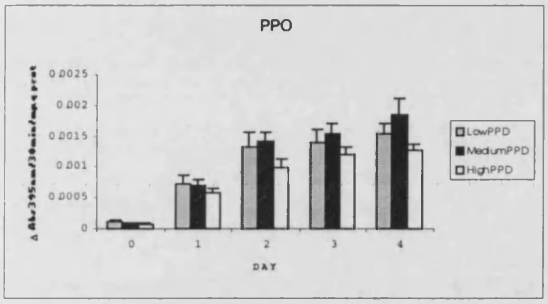


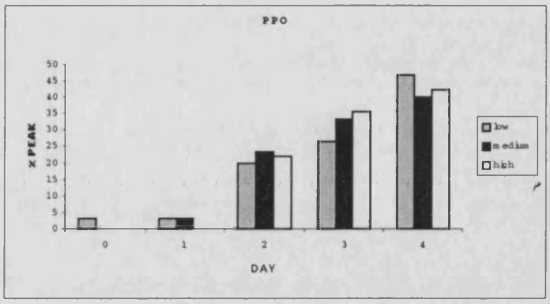


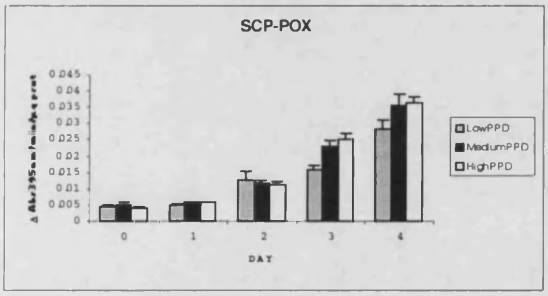
8.4.4 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in Family K samples group

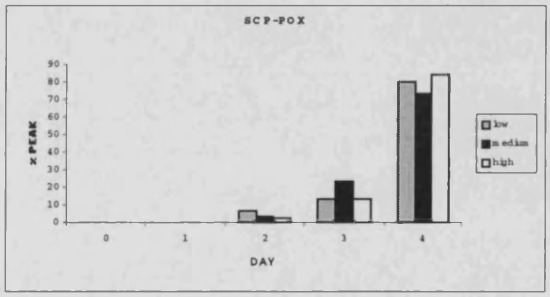


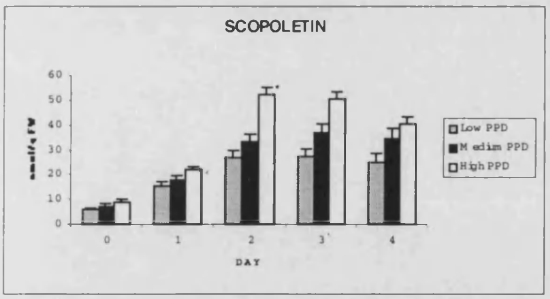


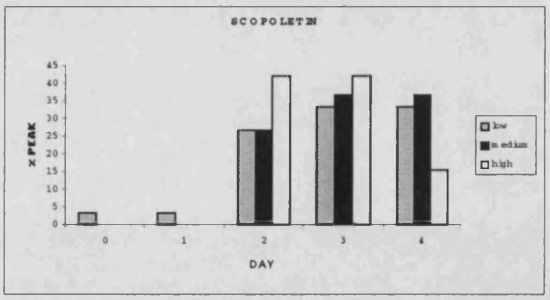




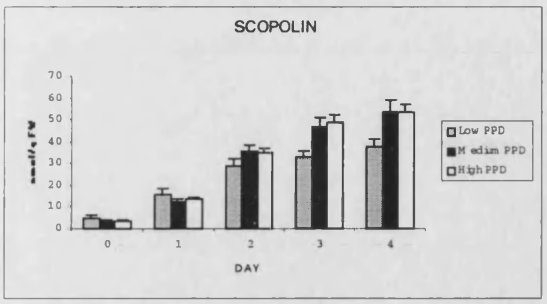


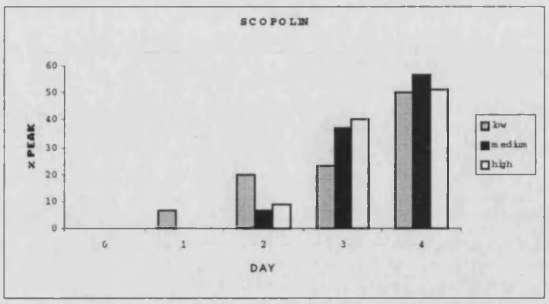


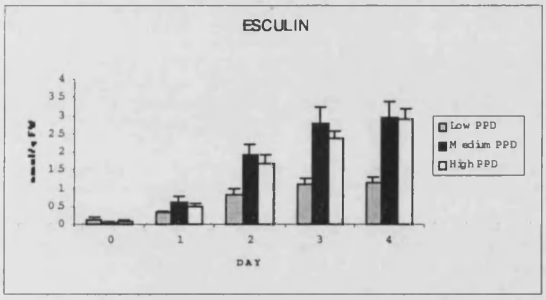


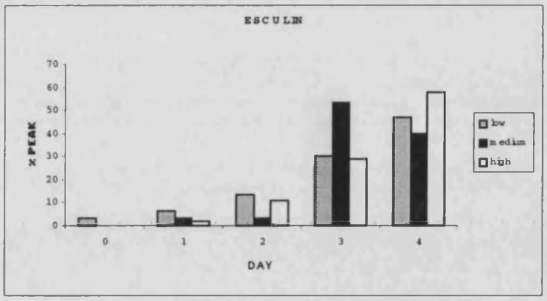


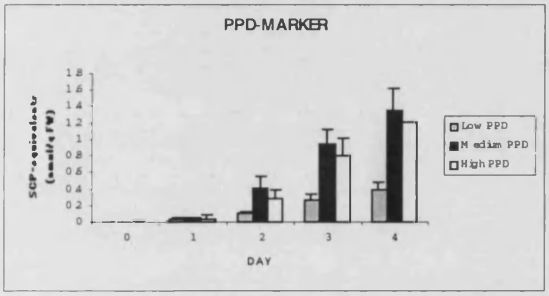
8.4.4 Graphs for average and peak frequency of secondary metabolites accumulation and enzymatic activity in Family K samples group

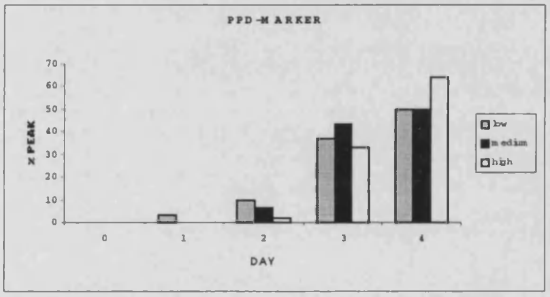


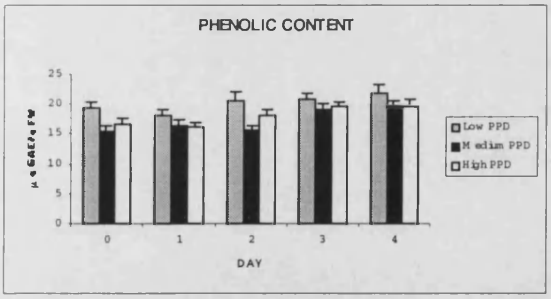


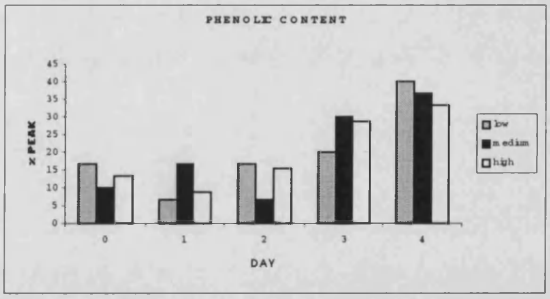












8.5 LINEAR CORRELATION ANALYSIS

A Pearson correlation analysis was also performed to study relationships among the different biochemical measurements, using the CORR procedure of SAS. Two dependent variables were considered, the measurement of each day and the AUTC. Correlations were studied for all cultivars and also among cultivars within PPD levels groups for each of the four groups of samples, Bath, June 1999, December 1999 and Family K.

The correlation coefficient measures the strength of the linear relationship between two variables. Correlations are always between -1 and 1 but can take any value in between. A positive correlation indicates that as one variable increases the other increases also. A negative correlation indicates that one variable increases as the other decreases.

Compared to visual inspection of a scatter plot, a correlation coefficient is a more reliable indicator of the strength of a linear relationship because the perception of a plot can be affected by its scale.

Correlation coefficients in bold are statistically significant. Correlation coefficients in bold and underline are also statistically significant but with $p <= 0.0001$.

8.5.1 Linear correlation analysis calculated by day

8.5.1.1 Linear correlation analysis calculated by day for Bath samples group

	all lev ESLIN	high ESLIN	low ESLIN	medium ESLIN		all lev ESTIN	high ESTIN	low ESTIN	medium ESTIN
ESLIN	1	1	1	1	ESLIN	0.10548	0.04287	0.25886	0.07451
	0	0	0	0		0.1736	0.6783	0.2219	0.6147
ESTIN	0.10548	0.04287	0.25886	0.07451	ESTIN	1	1	1	1
	0.1736	0.6783	0.2219	0.6147		0	0	0	0
SLIN	0.09674	0.0487	0.65168	0.20108	SLIN	0.08407	0.0773	0.18007	0.16424
	0.2122	0.6375	0.0006	0.1706		0.2786	0.4541	0.3998	0.2646
SCP	**0.37019**	**0.3709**	0.38975	-0.27061	SCP	**0.25223**	**0.29147**	0.00785	0.11102
	0.0001	0.0002	0.0597	0.0628		0.001	0.004	0.971	0.4525
G-CATE	**0.28412**	**0.35713**	0.04722	0.08361	G-CATE	0.0932	0.04939	-0.01842	**0.70742**
	0.0002	0.0004	0.8266	0.5721		0.2295	0.6328	0.9319	0.0001
CATE	**0.35906**	**0.43197**	**0.42192**	-0.04933	CATE	0.15032	0.10818	0.15456	0.14588
	0.0001	0.0001	0.04	0.7392		0.0518	0.2941	0.4708	0.3225
CATE-G	0.01707	-0.03838	0.19196	-0.06437	CATE-G	**0.18271**	**0.2076**	-0.00283	0.1239
	0.8262	0.7104	0.3689	0.6638		0.0178	0.0424	0.9895	0.4015

	all lev SLIN	high SLIN	low SLIN	medium SLIN		all lev SCP	high SCP	low SCP	medium SCP
ESLIN	0.09674	0.0487	**0.65168**	0.20108	ESLIN	**0.37019**	**0.3709**	0.38975	-0.27061
	0.2122	0.6375	0.0006	0.1706		0.0001	0.0002	0.0597	0.0628
ESTIN	0.08407	0.0773	0.18007	0.16424	ESTIN	**0.25223**	**0.29147**	0.00785	0.11102
	0.2786	0.4541	0.3998	0.2646		0.001	0.004	0.971	0.4525
SLIN	1	1	1	1	SLIN	**0.31879**	**0.30616**	**0.74234**	0.12413
	0	0	0	0		0.0001	0.0024	0.0001	0.4006
SCP	**0.31879**	**0.30616**	**0.74234**	0.12413	SCP	1	1	1	1
	0.0001	0.0024	0.0001	0.4006		0	0	0	0
G-CATE	**0.26949**	**0.28706**	0.30625	**0.4254**	G-CATE	**0.35811**	**0.49681**	0.12276	-0.0098
	0.0004	0.0046	0.1455	0.0026		0.0001	0.0001	0.5677	0.9473
CATE	**0.32444**	0.18696	**0.54437**	**0.5837**	CATE	**0.32959**	**0.29077**	**0.67048**	0.07214
	0.0001	0.0682	0.006	0.0001		0.0001	0.0041	0.0003	0.6261
CATE-G	**0.30769**	**0.37002**	0.19396	**0.29182**	CATE-G	**0.32276**	**0.37171**	0.15669	0.1954
	0.0001	0.0002	0.3638	0.0442		0.0001	0.0002	0.4647	0.1832

8.5.1.1 Linear correlation analysis calculated by day for Bath samples group

	all lev G-CATE	high G-CATE	low G-CATE	medium G-CATE
ESLIN	**0.28412**	**0.35713**	0.04722	0.08361
	0.0002	0.0004	0.8266	0.5721
ESTIN	0.0932	0.04939	-0.01842	**0.70742**
	0.2295	0.6328	0.9319	0.0001
SLIN	**0.26949**	**0.28706**	0.30625	**0.4254**
	0.0004	0.0046	0.1455	0.0026
SCP	**0.35811**	**0.49681**	**0.12276**	-0.0098
	0.0001	0.0001	0.5677	0.9473
G-CATE	1	1	1	1
	0	0	0	0
CATE	**0.34858**	**0.26017**	0.38082	**0.45222**
	0.0001	0.0105	0.0664	0.0013
CATE-G	**0.42115**	**0.38834**	**0.53384**	-0.02969
	0.0001	0.0001	0.0072	0.8412

	all lev CATE	high CATE	low CATE	medium CATE
ESLIN	**0.35906**	**0.43197**	**0.42192**	-0.04933
	0.0001	0.0001	0.04	0.7392
ESTIN	0.15032	0.10818	0.15456	0.14588
	0.0518	0.2941	0.4708	0.3225
SLIN	**0.32444**	0.18696	**0.54437**	**0.5837**
	0.0001	0.0682	0.006	0.0001
SCP	**0.32959**	**0.29077**	**0.67048**	0.07214
	0.0001	0.0041	0.0003	0.6261
G-CATE	**0.34858**	**0.26017**	0.38082	**0.45222**
	0.0001	0.0105	0.0664	0.0013
CATE	1	1	1	1
	0	0	0	0
CATE-G	**0.25053**	0.07104	**0.4567**	**0.45686**
	0.0011	0.4916	0.0249	0.0011

	all lev CATE-G	high CATE-G	low CATE-G	medium CATE-G
ESLIN	0.01707	-0.03838	0.19196	-0.06437
	0.8262	0.7104	0.3689	0.6638
ESTIN	**0.18271**	**0.2076**	-0.00283	0.1239
	0.0178	0.0424	0.9895	0.4015
SLIN	**0.30769**	**0.37002**	0.19396	**0.29182**
	0.0001	0.0002	0.3638	0.0442
SCP	**0.32276**	**0.37171**	**0.15669**	0.1954
	0.0001	0.0002	0.4647	0.1832
G-CATE	**0.42115**	**0.38834**	**0.53384**	-0.02969
	0.0001	0.0001	0.0072	0.8412
CATE	**0.25053**	0.07104	**0.4567**	**0.45686**
	0.0011	0.4916	0.0249	0.0011
CATE-G	1	1	1	1
	0	0	0	0

8.5.1.2 Linear correlation analysis calculated by day for June 1999 samples group

	all lev POX	high POX	low POX	medium POX		all lev PPO	high PPO	low PPO	medium PPO
POX	1	1	1	1	**POX**	**0.66304**	**0.84036**	**0.47635**	**0.5192**
	0	0	0	0		0.0001	0.0001	0.0006	0.0002
PPO	**0.66304**	**0.84036**	**0.47635**	**0.5192**	**PPO**	1	1	1	1
	0.0001	0.0001	0.0006	0.0002		0	0	0	0
CAT	0.09943	-0.0841	**0.56241**	0.09352	**CAT**	-0.09799	-0.2284	**0.51188**	0.2243
	0.2358	0.5698	0.0001	0.5272		0.2443	0.1225	0.0002	0.1254
PAL	**0.22802**	0.16229	**0.38661**	0.2445	**PAL**	0.12116	0.11567	0.02315	**0.52751**
	0.006	0.2704	0.0066	0.094		0.1495	0.4388	0.8759	0.0001
GLUC	**0.57617**	**0.6365**	**0.45331**	**0.47379**	**GLUC**	**0.60142**	**0.66384**	-0.20141	**0.56379**
	0.0001	0.0001	0.0012	0.0008		0.0001	0.0001	0.1698	0.0001
CHIT	**0.47481**	**0.73926**	0.26805	-0.11939	**CHIT**	**0.75202**	**0.79367**	**0.51888**	0.17854
	0.0001	0.0001	0.0655	0.4241		0.0001	0.0001	0.0002	0.2299
SCP	**0.19371**	**0.33723**	0.10276	-0.07144	**SCP**	**0.18996**	0.22489	**0.34974**	**-0.35034**
	0.02	0.0191	0.487	0.6294		0.0231	0.1286	0.0148	0.0146
SLIN	**0.3405**	**0.32541**	0.03815	**0.38948**	**SLIN**	**0.26425**	0.28098	0.0408	-0.10585
	0.0001	0.024	0.7968	0.0062		0.0014	0.0557	0.7831	0.474
PPD-M	**0.25347**	**0.6748**	**0.44023**	-0.06081	**PPD-M**	**0.26235**	**0.68712**	0.21629	-0.12162
	0.0022	0.0001	0.0017	0.6814		0.0015	0.0001	0.1398	0.4102
PHEN	**-0.17829**	-0.18397	**-0.44283**	**-0.39931**	**PHEN**	-0.08203	-0.25655	-0.16092	**-0.51109**
	0.0325	0.2107	0.0016	0.0049		0.3301	0.0817	0.2746	0.0002
TANNIN	-0.06612	-0.17307	-0.03564	-0.28019	**TANNIN**	0.01515	-0.22971	-0.05979	0.14393
	0.4311	0.2395	0.81	0.0537		0.8574	0.1204	0.6865	0.3291

	all lev CAT	high CAT	low CAT	medium CAT		all lev PAL	high PAL	low PAL	medium PAL
POX	0.09943	-0.0841	**0.56241**	0.09352	**POX**	**0.22802**	0.16229	**0.38661**	0.2445
	0.2358	0.5698	0.0001	0.5272		0.006	0.2704	0.0066	0.094
PPO	-0.09799	-0.2284	**0.51188**	0.2243	**PPO**	0.12116	0.11567	0.02315	**0.52751**
	0.2443	0.1225	0.0002	0.1254		0.1495	0.4388	0.8759	0.0001
CAT	1	1	1	1	**CAT**	**0.207**	-0.0892	-0.02282	0.2347
	0	0	0	0		0.0128	0.5466	0.8776	0.1083
PAL	**0.207**	-0.0892	-0.02282	0.2347	**PAL**	1	1	1	1
	0.0128	0.5466	0.8776	0.1083		0	0	0	0
GLUC	-0.05963	-0.25097	0.11865	0.10608	**GLUC**	**0.20966**	0.25114	**0.44556**	0.18659
	0.484	0.0963	0.4219	0.4779		0.0129	0.0961	0.0015	0.2092
CHIT	-0.04389	-0.26308	**0.46696**	**0.29322**	**CHIT**	**0.18412**	0.06252	-0.10294	**0.41406**
	0.6053	0.0773	0.0008	0.0455		0.0288	0.6798	0.4863	0.0038
SCP	-0.0584	0.13232	0.03272	**-0.3251**	**SCP**	-0.12579	0.21643	-0.03013	**-0.46909**
	0.4869	0.37	0.8253	0.0242		0.133	0.1395	0.8389	0.0008
SLIN	0.02771	0.14881	0.05994	-0.10531	**SLIN**	-0.03237	0.23037	-0.03977	**-0.29431**
	0.7416	0.3127	0.6857	0.4763		0.7001	0.1152	0.7884	0.0423
PPD-M	0.01077	-0.11468	0.22735	-0.02792	**PPD-M**	0.01386	**0.48935**	0.24898	-0.20521
	0.8981	0.4377	0.1202	0.8506		0.8691	0.0004	0.0879	0.1617
PHEN	**-0.29624**	-0.27864	**-0.39883**	**-0.45331**	**PHEN**	**-0.19962**	0.02047	-0.09885	**-0.54012**
	0.0003	0.0551	0.005	0.0012		0.0165	0.8902	0.5039	0.0001
TANNIN	-0.14301	-0.23404	-0.08973	-0.02804	**TANNIN**	**0.19001**	0.03756	-0.23689	**0.66673**
	0.0873	0.1094	0.5442	0.8499		0.0225	0.7999	0.105	0.0001

8.5.1.2 Linear correlation analysis calculated by day for June 1999 samples group

	all lev GLUC	high GLUC	low GLUC	medium GLUC		all lev CHIT	high CHIT	low CHIT	medium CHIT
POX	**0.57617**	**0.6365**	**0.45331**	**0.47379**	**POX**	**0.47481**	**0.73926**	0.26805	-0.11939
	0.0001	0.0001	0.0012	0.0008		0.0001	0.0001	0.0655	0.4241
PPO	**0.60142**	**0.66384**	-0.20141	**0.56379**	**PPO**	**0.75202**	**0.79367**	**0.51888**	0.17854
	0.0001	0.0001	0.1698	0.0001		0.0001	0.0001	0.0002	0.2299
CAT	-0.05963	-0.25097	0.11865	0.10608	**CAT**	-0.04389	-0.26308	**0.46696**	**0.29322**
	0.484	0.0963	0.4219	0.4779		0.6053	0.0773	0.0008	0.0455
PAL	**0.20966**	0.25114	**0.44556**	0.18659	**PAL**	**0.18412**	0.06252	-0.10294	**0.41406**
	0.0129	0.0961	0.0015	0.2092		0.0288	0.6798	0.4863	0.0038
GLUC	1	1	1	1	**GLUC**	**0.3871**	**0.47603**	-0.07695	-0.12689
	0	0	0	0		0.0001	0.0009	0.6032	0.3954
CHIT	**0.3871**	**0.47603**	-0.07695	-0.12689	**CHIT**	1	1	1	1
	0.0001	0.0009	0.6032	0.3954		0	0	0	0
SCP	0.0944	0.13279	-0.08374	-0.14262	**SCP**	0.06005	0.1178	-0.00112	**-0.42695**
	0.2673	0.3845	0.5715	0.3389		0.4794	0.4356	0.994	0.0028
SLIN	**0.21921**	0.18045	0.10799	0.08755	**SLIN**	0.13269	0.14458	0.08115	**-0.31624**
	0.0093	0.2356	0.465	0.5584		0.1168	0.3377	0.5835	0.0303
PPD-M	**0.25905**	**0.524**	**0.50597**	-0.05357	**PPD-M**	**0.1876**	**0.56187**	0.02906	-0.1781
	0.002	0.0002	0.0002	0.7206		0.0259	0.0001	0.8446	0.231
PHEN	-0.05695	-0.07674	**-0.41871**	-0.27553	**PHEN**	-0.07086	-0.16493	-0.0806	**-0.42518**
	0.5039	0.6163	0.0031	0.0609		0.4037	0.2734	0.586	0.0029
TANNIN	0.10984	0.02424	-0.04064	-0.08321	**TANNIN**	0.07105	-0.16599	0.16752	**0.32283**
	0.1964	0.8744	0.7839	0.5782		0.4025	0.2703	0.2551	0.0269

	all lev SCP	high SCP	low SCP	medium SCP		all lev SLIN	high SLIN	low SLIN	medium SLIN
POX	**0.19371**	**0.33723**	0.10276	-0.07144	**POX**	**0.3405**	**0.32541**	0.03815	**0.38948**
	0.02	0.0191	0.487	0.6294		0.0001	0.024	0.7968	0.0062
PPO	**0.18996**	0.22489	**0.34974**	**-0.35034**	**PPO**	**0.26425**	0.28098	0.0408	-0.10585
	0.0231	0.1286	0.0148	0.0146		0.0014	0.0557	0.7831	0.474
CAT	-0.0584	0.13232	0.03272	**-0.3251**	**CAT**	0.02771	0.14881	0.05994	-0.10531
	0.4869	0.37	0.8253	0.0242		0.7416	0.3127	0.6857	0.4763
PAL	-0.12579	0.21643	-0.03013	**-0.46909**	**PAL**	-0.03237	0.23037	-0.03977	**-0.29431**
	0.133	0.1395	0.8389	0.0008		0.7001	0.1152	0.7884	0.0423
GLUC	0.0944	0.13279	-0.08374	-0.14262	**GLUC**	**0.21921**	0.18045	0.10799	0.08755
	0.2673	0.3845	0.5715	0.3389		0.0093	0.2356	0.465	0.5584
CHIT	0.06005	0.1178	-0.00112	**-0.42695**	**CHIT**	0.13269	0.14458	0.08115	**-0.31624**
	0.4794	0.4356	0.994	0.0028		0.1168	0.3377	0.5835	0.0303
SCP	1	1	1	1	**SCP**	**0.65304**	**0.7056**	**0.32972**	**0.6982**
	0	0	0	0		0.0001	0.0001	0.0221	0.0001
SLIN	**0.65304**	**0.7056**	**0.32972**	**0.6982**	**SLIN**	1	1	1	1
	0.0001	0.0001	0.0221	0.0001		0	0	0	0
PPD-M	**0.28325**	**0.37522**	0.15466	0.28195	**PPD-M**	**0.30195**	**0.42372**	0.24317	**0.28949**
	0.0006	0.0086	0.2939	0.0522		0.0002	0.0027	0.0958	0.046
PHEN	**0.19004**	-0.25008	-0.02037	**0.67612**	**PHEN**	0.07152	-0.21251	-0.21926	0.23731
	0.0225	0.0865	0.8907	0.0001		0.3943	0.147	0.1343	0.1044
TANNIN	**-0.17354**	**-0.36279**	-0.27793	-0.15721	**TANNIN**	-0.1003	**-0.30303**	-0.02322	**-0.34703**
	0.0375	0.0113	0.0558	0.2859		0.2317	0.0363	0.8755	0.0157

8.5.1.2 Linear correlation analysis calculated by day for June 1999 samples group

	all lev PPD-M	high PPD-M	low PPD-M	medium PPD-M
POX	**0.25347**	**0.6748**	**0.44023**	-0.06081
	0.0022	0.0001	0.0017	0.6814
PPO	**0.26235**	**0.68712**	0.21629	-0.12162
	0.0015	0.0001	0.1398	0.4102
CAT	0.01077	-0.11468	0.22735	-0.02792
	0.8981	0.4377	0.1202	0.8506
PAL	0.01386	**0.48935**	0.24898	-0.20521
	0.8691	0.0004	0.0879	0.1617
GLUC	**0.25905**	**0.524**	**0.50597**	-0.05357
	0.002	0.0002	0.0002	0.7206
CHIT	**0.1876**	**0.56187**	0.02906	-0.1781
	0.0259	0.0001	0.8446	0.231
SCP	**0.28325**	**0.37522**	0.15466	0.28195
	0.0006	0.0086	0.2939	0.0522
SLIN	**0.30195**	**0.42372**	0.24317	**0.28949**
	0.0002	0.0027	0.0958	0.046
PPD-M	1	1	1	1
	0	0	0	0
PHEN	0.11621	-0.02065	**-0.34**	**0.30351**
	0.1654	0.8892	0.0181	0.036
TANNIN	-0.04726	-0.12825	-0.00882	0.00736
	0.5738	0.385	0.9525	0.9604

	all lev PHEN	high PHEN	low PHEN	medium PHEN
POX	**-0.17829**	-0.18397	**-0.44283**	**-0.39931**
	0.0325	0.2107	0.0016	0.0049
PPO	-0.08203	-0.25655	-0.16092	**-0.51109**
	0.3301	0.0817	0.2746	0.0002
CAT	**-0.29624**	-0.27864	**-0.39883**	**-0.45331**
	0.0003	0.0551	0.005	0.0012
PAL	**-0.19962**	0.02047	-0.09885	**-0.54012**
	0.0165	0.8902	0.5039	0.0001
GLUC	-0.05695	-0.07674	**-0.41871**	-0.27553
	0.5039	0.6163	0.0031	0.0609
CHIT	-0.07086	-0.16493	-0.0806	**-0.42518**
	0.4037	0.2734	0.586	0.0029
SCP	**0.19004**	-0.25008	-0.02037	**0.67612**
	0.0225	0.0865	0.8907	0.0001
SLIN	0.07152	-0.21251	-0.21926	0.23731
	0.3943	0.147	0.1343	0.1044
PPD-M	0.11621	-0.02065	**-0.34**	**0.30351**
	0.1654	0.8892	0.0181	0.036
PHEN	1	1	1	1
	0	0	0	0
TANNIN	**0.65044**	**0.84566**	0.10372	0.05116
	0.0001	0.0001	0.483	0.7298

	all lev TANNIN	high TANNIN	low TANNIN	medium TANNIN
POX	-0.06612	-0.17307	-0.03564	-0.28019
	0.4311	0.2395	0.81	0.0537
PPO	0.01515	-0.22971	-0.05979	0.14393
	0.8574	0.1204	0.6865	0.3291
CAT	-0.14301	-0.23404	-0.08973	-0.02804
	0.0873	0.1094	0.5442	0.8499
PAL	**0.19001**	0.03756	-0.23689	**0.66673**
	0.0225	0.7999	0.105	0.0001
GLUC	0.10984	0.02424	-0.04064	-0.08321
	0.1964	0.8744	0.7839	0.5782
CHIT	0.07105	-0.16599	0.16752	**0.32283**
	0.4025	0.2703	0.2551	0.0269
SCP	**-0.17354**	**-0.36279**	-0.27793	-0.15721
	0.0375	0.0113	0.0558	0.2859
SLIN	-0.1003	**-0.30303**	-0.02322	**-0.34703**
	0.2317	0.0363	0.8755	0.0157
PPD-M	-0.04726	-0.12825	-0.00882	0.00736
	0.5738	0.385	0.9525	0.9604
PHEN	**0.65044**	**0.84566**	0.10372	0.05116
	0.0001	0.0001	0.483	0.7298
TANNIN	1	1	1	1
	0	0	0	0

8.5.1.3 *Linear correlation analysis calculated by day for December1999 samples group*

	all lev POX	high POX	low POX	medium POX		all lev SCPOX	high SCPOX	low SCPOX	medium SCPOX
POX	1	1	1	1	**POX**	**0.16233**	**0.28509**	0.14516	0.20437
	0	0	0	0		0.0472	0.0273	0.3414	0.1781
SCPOX	**0.16233**	**0.28509**	0.14516	0.20437	**SCPOX**	1	1	1	1
	0.0472	0.0273	0.3414	0.1781		0	0	0	0
PPO	**0.38331**	0.13296	**0.60642**	-0.03026	**PPO**	**0.3569**	**0.64206**	**0.382**	0.2432
	0.0001	0.3112	0.0001	0.8436		0.0001	0.0001	0.0096	0.1074
CAT	**0.49542**	**0.38931**	**0.50792**	**0.61529**	**CAT**	**0.19413**	**0.3007**	**0.42109**	0.02406
	0.0001	0.0021	0.0004	0.0001		0.0173	0.0196	0.004	0.8753
SCP	0.04914	0.18803	-0.01345	0.05895	**SCP**	**0.36468**	**0.39282**	0.18467	**0.36246**
	0.5504	0.1502	0.9301	0.7005		0.0001	0.0019	0.2246	0.0144
SLIN	-0.0221	0.00132	0.02022	-0.13536	**SLIN**	**0.52103**	**0.68966**	**0.45968**	**0.26311**
	0.7883	0.992	0.8951	0.3753		0.0001	0.0001	0.0015	0.0808
ESLIN	**0.19655**	**0.49599**	**0.31638**	-0.05662	**ESLIN**	**0.54774**	**0.65259**	**0.58384**	**0.41216**
	0.0159	0.0001	0.0342	0.7118		0.0001	0.0001	0.0001	0.0049
PPD-M	**0.16181**	0.24143	0.17982	0.19514	**PPD-M**	**0.69304**	**0.77922**	**0.53565**	**0.62374**
	0.0479	0.0631	0.2372	0.1989		0.0001	0.0001	0.0001	0.0001
PHEN	**-0.20536**	-0.09659	-0.23119	-0.2457	**PHEN**	**0.18178**	0.01778	**0.38006**	**0.3229**
	0.0117	0.4628	0.1265	0.1038		0.026	0.8928	0.01	0.0305

	all lev PPO	high PPO	low PPO	medium PPO		all lev CAT	high CAT	low CAT	medium CAT
POX	**0.38331**	0.13296	**0.60642**	-0.03026	**POX**	**0.49542**	**0.38931**	**0.50792**	**0.61529**
	0.0001	0.3112	0.0001	0.8436		0.0001	0.0021	0.0004	0.0001
SCPOX	**0.3569**	**0.64206**	**0.382**	0.2432	**SCPOX**	**0.19413**	**0.3007**	**0.42109**	0.02406
	0.0001	0.0001	0.0096	0.1074		0.0173	0.0196	0.004	0.8753
PPO	1	1	1	1	**PPO**	**0.36997**	**0.35333**	**0.60431**	-0.00558
	0	0	0	0		0.0001	0.0056	0.0001	0.971
CAT	**0.36997**	**0.35333**	**0.60431**	-0.00558	**CAT**	1	1	1	1
	0.0001	0.0056	0.0001	0.971		0	0	0	0
SCP	**0.24879**	**0.33667**	0.25996	**0.38632**	**SCP**	0.15498	**0.39057**	0.20429	-0.05348
	0.0021	0.0085	0.0846	0.0088		0.0583	0.002	0.1783	0.7272
SLIN	**0.47759**	**0.70705**	0.22812	**0.44177**	**SLIN**	**0.21883**	0.24156	**0.36164**	0.13227
	0.0001	0.0001	0.1318	0.0024		0.0071	0.063	0.0147	0.3864
ESLIN	**0.33298**	**0.4058**	**0.46395**	**0.44461**	**ESLIN**	0.15428	**0.2636**	**0.55723**	-0.02187
	0.0001	0.0013	0.0013	0.0022		0.0594	0.0418	0.0001	0.8866
PPD-M	**0.37025**	**0.45079**	**0.40817**	**0.49719**	**PPD-M**	**0.23887**	**0.29077**	**0.37765**	0.13885
	0.0001	0.0003	0.0054	0.0005		0.0032	0.0242	0.0105	0.363
PHEN	**-0.1978**	-0.2124	-0.14342	-0.06513	**PHEN**	**-0.23062**	-0.18659	-0.11086	**-0.42779**
	0.0153	0.1033	0.3473	0.6708		0.0045	0.1534	0.4685	0.0034

8.5.1.3 Linear correlation analysis calculated by day for December1999 samples group

	all lev SCP	high SCP	low SCP	medium SCP		all lev SLIN	high SLIN	low SLIN	medium SLIN
POX	0.04914	0.18803	-0.01345	0.05895	**POX**	-0.0221	0.00132	0.02022	-0.13536
	0.5504	0.1502	0.9301	0.7005		0.7883	0.992	0.8951	0.3753
SCPOX	**0.36468**	**0.39282**	0.18467	**0.36246**	**SCPOX**	**0.52103**	**0.68966**	**0.45968**	0.26311
	0.0001	0.0019	0.2246	0.0144		0.0001	0.0001	0.0015	0.0808
PPO	**0.24879**	**0.33667**	0.25996	**0.38632**	**PPO**	**0.47759**	**0.70705**	0.22812	**0.44177**
	0.0021	0.0085	0.0846	0.0088		0.0001	0.0001	0.1318	0.0024
CAT	0.15498	**0.39057**	0.20429	-0.05348	**CAT**	0.21883	0.24156	**0.36164**	0.13227
	0.0583	0.002	0.1783	0.7272		0.0071	0.063	0.0147	0.3864
SCP	1	1	1	1	**SCP**	**0.44857**	**0.45314**	**0.51699**	**0.4666**
	0	0	0	0		0.0001	0.0003	0.0003	0.0012
SLIN	**0.44857**	**0.45314**	**0.51699**	**0.4666**	**SLIN**	1	1	1	1
	0.0001	0.0003	0.0003	0.0012		0	0	0	0
ESLIN	**0.43793**	**0.4139**	**0.46692**	**0.5266**	**ESLIN**	**0.44572**	0.41238	**0.69295**	**0.41182**
	0.0001	0.001	0.0012	0.0002		0.0001	0.0011	0.0001	0.0049
PPD-M	**0.44649**	**0.33737**	**0.36713**	**0.57239**	**PPD-M**	**0.5444**	**0.64385**	0.25696	**0.3988**
	0.0001	0.0084	0.0131	0.0001		0.0001	0.0001	0.0884	0.0067
PHEN	0.03479	0.01244	-0.00924	0.16137	**PHEN**	0.0303	-0.13447	**0.40042**	0.10861
	0.6725	0.9248	0.952	0.2896		0.7128	0.3057	0.0064	0.4776

	all lev ESLIN	high ESLIN	low ESLIN	medium ESLIN		all lev PPD-M	high PPD-M	low PPD-M	medium PPD-M
POX	**0.19655**	**0.49599**	**0.31638**	-0.05662	**POX**	**0.16181**	0.24143	0.17982	0.19514
	0.0159	0.0001	0.0342	0.7118		0.0479	0.0631	0.2372	0.1989
SCPOX	**0.54774**	**0.65259**	**0.58384**	**0.41216**	**SCPOX**	**0.69304**	**0.77922**	**0.53565**	**0.62374**
	0.0001	0.0001	0.0001	0.0049		0.0001	0.0001	0.0001	0.0001
PPO	**0.33298**	**0.4058**	**0.46395**	**0.44461**	**PPO**	**0.37025**	**0.45079**	**0.40817**	**0.49719**
	0.0001	0.0013	0.0013	0.0022		0.0001	0.0003	0.0054	0.0005
CAT	0.15428	**0.2636**	**0.55723**	-0.02187	**CAT**	**0.23887**	**0.29077**	**0.37765**	0.13885
	0.0594	0.0418	0.0001	0.8866		0.0032	0.0242	0.0105	0.363
SCP	**0.43793**	**0.4139**	**0.46692**	**0.5266**	**SCP**	**0.44649**	**0.33737**	**0.36713**	**0.57239**
	0.0001	0.001	0.0012	0.0002		0.0001	0.0084	0.0131	0.0001
SLIN	**0.44572**	**0.41238**	**0.69295**	**0.41182**	**SLIN**	**0.5444**	**0.64385**	0.25696	**0.3988**
	0.0001	0.0011	0.0001	0.0049		0.0001	0.0001	0.0884	0.0067
ESLIN	1	1	1	1	**ESLIN**	**0.67805**	**0.62548**	**0.65952**	**0.79225**
	0	0	0	0		0.0001	0.0001	0.0001	0.0001
PPD-M	**0.67805**	**0.62548**	**0.65952**	**0.79225**	**PPD-M**	1	1	1	1
	0.0001	0.0001	0.0001	0.0001		0	0	0	0
PHEN	0.03282	-0.00116	0.12255	0.27782	**PHEN**	-0.00595	0.00574	-0.11797	0.21664
	0.6901	0.993	0.4226	0.0646		0.9424	0.9653	0.4402	0.1529

8.5.1.3 Linear correlation analysis calculated by day for December1999 samples group

	all lev PHEN	high PHEN	low PHEN	medium PHEN
POX	-0.20536	-0.09659	-0.23119	-0.2457
	0.0117	0.4628	0.1265	0.1038
SCPOX	**0.18178**	0.01778	**0.38006**	**0.3229**
	0.026	0.8928	0.01	0.0305
PPO	**-0.1978**	-0.2124	-0.14342	-0.06513
	0.0153	0.1033	0.3473	0.6708
CAT	**-0.23062**	-0.18659	-0.11086	-0.42779
	0.0045	0.1534	0.4685	0.0034
SCP	0.03479	0.01244	-0.00924	0.16137
	0.6725	0.9248	0.952	0.2896
SLIN	0.0303	-0.13447	**0.40042**	0.10861
	0.7128	0.3057	0.0064	0.4776
ESLIN	0.03282	-0.00116	0.12255	0.27782
	0.6901	0.993	0.4226	0.0646
PPD-M	-0.00595	0.00574	-0.11797	0.21664
	0.9424	0.9653	0.4402	0.1529
PHEN	1	1	1	1
	0	0	0	0

8.5.1.4 Linear correlation analysis calculated by day for Family K samples group

	all lev POX	low POX	medium POX	high POX		all lev SCPOX	low SCPOX	medium SCPOX	high SCPOX
POX	1	1	1	1	**POX**	-0.0049	**0.39662**	**0.28057**	-0.0126
	0	0	0	0		0.9108	0.0001	0.0005	0.8509
SCPOX	-0.0049	**0.39662**	**0.28057**	-0.0126	**SCPOX**	1	1	1	1
	0.9108	0.0001	0.0005	0.8509		0	0	0	0
PPO	0.01584	0.00341	**0.18974**	0.0423	**PPO**	**0.35078**	**0.19052**	**0.37423**	**0.50649**
	0.7173	0.967	0.02	0.5278		0.0001	0.0195	0.0001	0.0001
SCP	0.04775	**0.18532**	0.00661	0.04734	**SCP**	**0.34441**	**0.30949**	**0.40449**	**0.31072**
	0.2748	0.0232	0.9361	0.4799		0.0001	0.0001	0.0001	0.0001
SLIN	0.04039	**0.27742**	**0.22947**	0.0534	**SLIN**	**0.59299**	**0.44122**	**0.64229**	**0.62041**
	0.3557	0.0006	0.0047	0.4254		0.0001	0.0001	0.0001	0.0001
ESLIN	-0.00599	**0.40586**	0.13532	-0.01694	**ESLIN**	**0.61245**	**0.52049**	**0.59122**	**0.70024**
	0.8914	0.0001	0.0999	0.8009		0.0001	0.0001	0.0001	0.0001
PPD-M	0.00911	**0.28335**	0.11971	0.00772	**PPD-M**	**0.62793**	**0.58676**	**0.71404**	**0.62204**
	0.835	0.0004	0.1445	0.9084		0.0001	0.0001	0.0001	0.0001
PHEN	-0.04272	-0.05832	-0.06043	-0.06168	**PHEN**	**0.16362**	0.14736	**0.23757**	**0.17527**
	0.3291	0.4799	0.4626	0.3571		0.0002	0.0729	0.0034	0.0084

	all lev PPO	low PPO	medium PPO	high PPO		all lev SCP	low SCP	medium SCP	high SCP
POX	0.01584	0.00341	**0.18974**	0.0423	**POX**	0.04775	**0.18532**	0.00661	0.04734
	0.7173	0.967	0.02	0.5278		0.2748	0.0232	0.9361	0.4799
SCPOX	**0.35078**	**0.19052**	**0.37423**	**0.50649**	**SCPOX**	**0.34441**	**0.30949**	**0.40449**	**0.31072**
	0.0001	0.0195	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001
PPO	1	1	1	1	**PPO**	**0.0971**	-0.02403	0.15268	0.23177
	0	0	0	0		0.0261	0.7704	0.0621	0.0005
SCP	**0.0971**	-0.02403	0.15268	**0.23177**	**SCP**	1	1	1	1
	0.0261	0.7704	0.0621	0.0005		0	0	0	0
SLIN	**0.39659**	**0.35548**	**0.43588**	**0.4517**	**SLIN**	**0.66566**	**0.5778**	**0.70095**	**0.68602**
	0.0001	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001
ESLIN	**0.28853**	**0.16362**	**0.25931**	**0.47751**	**ESLIN**	**0.3964**	**0.55048**	**0.31261**	**0.40663**
	0.0001	0.0454	0.0014	0.0001		0.0001	0.0001	0.0001	0.0001
PPD-M	**0.2693**	0.13887	**0.29159**	**0.3841**	**PPD-M**	**0.26294**	**0.43551**	**0.24517**	**0.21824**
	0.0001	0.0901	0.0003	0.0001		0.0001	0.0001	0.0025	0.001
PHEN	0.05605	0.0399	0.06339	0.07362	**PHEN**	**0.20643**	**0.23545**	**0.23569**	**0.27882**
	0.2002	0.629	0.4409	0.2715		0.0001	0.0038	0.0037	0.0001

8.5.1.4 Linear correlation analysis calculated by day for Family K samples group

	all lev SLIN	low SLIN	medium SLIN	high SLIN		all lev ESLIN	low ESLIN	medium ESLIN	high ESLIN
POX	0.04039	**0.27742**	**0.22947**	0.0534	**POX**	-0.00599	**0.40586**	0.13532	-0.01694
	0.3557	0.0006	0.0047	0.4254		0.8914	0.0001	0.0999	0.8009
SCPOX	**0.59299**	**0.44122**	**0.64229**	**0.62041**	**SCPOX**	**0.61245**	**0.52049**	**0.59122**	**0.70024**
	0.0001	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001
PPO	**0.39659**	**0.35548**	**0.43588**	**0.4517**	**PPO**	**0.28853**	**0.16362**	**0.25931**	**0.47751**
	0.0001	0.0001	0.0001	0.0001		0.0001	0.0454	0.0014	0.0001
SCP	**0.66566**	**0.5778**	**0.70095**	**0.68602**	**SCP**	**0.3964**	**0.55048**	**0.31261**	**0.40663**
	0.0001	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001
SLIN	1	1	1	1	**SLIN**	**0.59613**	**0.66675**	**0.50261**	**0.67354**
	0	0	0	0		0.0001	0.0001	0.0001	0.0001
ESLIN	**0.59613**	**0.66675**	**0.50261**	**0.67354**	**ESLIN**	1	1	1	1
	0.0001	0.0001	0.0001	0.0001		0	0	0	0
PPD-M	**0.4575**	**0.46695**	**0.46118**	**0.45751**	**PPD-M**	**0.70248**	**0.64167**	**0.72045**	**0.66563**
	0.0001	0.0001	0.0001	0.0001		0.0001	0.0001	0.0001	0.0001
PHEN	**0.15754**	**0.2521**	**0.18483**	**0.15097**	**PHEN**	**0.11975**	0.04513	**0.20855**	**0.20871**
	0.0003	0.0019	0.0236	0.0235		0.0062	0.5847	0.0107	0.0017

	all lev PPD-M	low PPD-M	medium PPD-M	high PPD-M		all lev PHEN	low PHEN	medium PHEN	high PHEN
POX	0.00911	**0.28335**	0.11971	0.00772	**POX**	-0.04272	-0.05832	-0.06043	-0.06168
	0.835	0.0004	0.1445	0.9084		0.3291	0.4799	0.4626	0.3571
SCPOX	**0.62793**	**0.58676**	**0.71404**	**0.62204**	**SCPOX**	**0.16362**	0.14736	**0.23757**	**0.17527**
	0.0001	0.0001	0.0001	0.0001		0.0002	0.0729	0.0034	0.0084
PPO	**0.2693**	0.13887	**0.29159**	**0.3841**	**PPO**	0.05605	0.0399	0.06339	0.07362
	0.0001	0.0901	0.0003	0.0001		0.2002	0.629	0.4409	0.2715
SCP	**0.26294**	**0.43551**	**0.24517**	**0.21824**	**SCP**	**0.20643**	**0.23545**	**0.23569**	**0.27882**
	0.0001	0.0001	0.0025	0.001		0.0001	0.0038	0.0037	0.0001
SLIN	**0.4575**	**0.46695**	**0.46118**	**0.45751**	**SLIN**	**0.15754**	**0.2521**	**0.18483**	**0.15097**
	0.0001	0.0001	0.0001	0.0001		0.0003	0.0019	0.0236	0.0235
ESLIN	**0.70248**	**0.64167**	**0.72045**	**0.66563**	**ESLIN**	**0.11975**	0.04513	**0.20855**	**0.20871**
	0.0001	0.0001	0.0001	0.0001		0.0062	0.5847	0.0107	0.0017
PPD-M	1	1	1	1	**PPD-M**	**0.09833**	0.08737	**0.19727**	**0.13873**
	0	0	0	0		0.0244	0.2893	0.0155	0.0376
PHEN	**0.09833**	0.08737	**0.19727**	**0.13873**	**PHEN**	1	1	1	1
	0.0244	0.2893	0.0155	0.0376		0	0	0	0

8.5.2 Linear correlation analysis calculated by area under the curve (AUTC)

8.5.2.1 Linear correlation analysis calculated by AUTC for Bath samples group

	all lev CATE	high CATE	low CATE	medium CATE		all lev CATE-G	high CATE-G	low CATE-G	medium CATE-G
CATE	1	1	1	1	**CATE**	0.14136	-0.10847	0.99153	0.53293
	0	0	0	0		0.5411	0.7372	0.0829	0.2763
CATE-G	0.14136	-0.10847	0.99153	0.53293	**CATE-G**	1	1	1	1
	0.5411	0.7372	0.0829	0.2763		0	0	0	0
ESTIN	**0.67167**	**0.61775**	**0.99764**	0.06355	**ESTIN**	**0.57352**	0.52834	0.98029	0.31825
	0.0009	0.0323	0.0437	0.9048		0.0066	0.0774	0.1266	0.5387
ESLIN	0.40862	0.4718	0.98318	0.10263	**ESLIN**	-0.04844	-0.13907	0.95114	0.00643
	0.0659	0.1215	0.1169	0.8466		0.8348	0.6664	0.1998	0.9904
G-CATE	0.21579	0.1003	-0.38111	0.2416	**G-CATE**	0.29844	0.25678	-0.25784	-0.039
	0.3475	0.7564	0.7511	0.6447		0.1888	0.4204	0.834	0.9415
SCP	0.18573	0.22305	0.59521	0.17193	**SCP**	0.3601	0.3358	0.48583	0.28388
	0.4202	0.4859	0.5941	0.7446		0.1088	0.2859	0.677	0.5856
SLIN	0.18828	-0.09601	0.77079	0.73674	**SLIN**	**0.51641**	**0.70687**	0.84699	-0.00511
	0.4137	0.7666	0.4397	0.0948		0.0165	0.0102	0.3568	0.9923

	all lev ESTIN	high ESTIN	low ESTIN	medium ESTIN		all lev ESLIN	high ESLIN	low ESLIN	medium ESLIN
CATE	**0.67167**	**0.61775**	**0.99764**	0.06355	**CATE**	0.40862	0.4718	0.98318	0.10263
	0.0009	0.0323	0.0437	0.9048		0.0659	0.1215	0.1169	0.8466
CATE-G	**0.57352**	0.52834	0.98029	0.31825	**CATE-G**	-0.04844	-0.13907	0.95114	0.00643
	0.0066	0.0774	0.1266	0.5387		0.8348	0.6664	0.1998	0.9904
ESTIN	1	1	1	1	**ESTIN**	0.15287	0.02159	0.99339	-0.792
	0	0	0	0		0.5083	0.9469	0.0732	0.0604
ESLIN	0.15287	0.02159	0.99339	-0.792	**ESLIN**	1	1	1	1
	0.5083	0.9469	0.0732	0.0604		0	0	0	0
G-CATE	0.17875	0.07992	-0.44365	**0.81172**	**G-CATE**	**0.52306**	0.56354	-0.54356	-0.7657
	0.4382	0.805	0.7074	0.0498		0.015	0.0564	0.6342	0.0759
SCP	0.29005	0.21072	0.64894	**0.96055**	**SCP**	**0.76311**	**0.75666**	0.73196	**-0.88498**
	0.2022	0.5109	0.5504	0.0023		0.0001	0.0044	0.4772	0.0191
SLIN	0.17042	0.24106	0.72526	-0.06336	**SLIN**	-0.0819	-0.10701	0.64147	0.08842
	0.4602	0.4504	0.4834	0.9051		0.7241	0.7406	0.5567	0.8677

8.5.2.1 Linear correlation analysis calculated by AUTC for Bath samples group

	all lev G-CATE	high G-CATE	low G-CATE	medium G-CATE
CATE	0.21579	0.1003	-0.38111	0.2416
	0.3475	0.7564	0.7511	0.6447
CATE-G	0.29844	0.25678	-0.25784	-0.039
	0.1888	0.4204	0.834	0.9415
ESTIN	0.17875	0.07992	-0.44365	**0.81172**
	0.4382	0.805	0.7074	0.0498
ESLIN	**0.52306**	0.56354	-0.54356	-0.7657
	0.015	0.0564	0.6342	0.0759
G-CATE	1	1	1	1
	0	0	0	0
SCP	**0.69404**	**0.85908**	-0.96977	**0.88638**
	0.0005	0.0003	0.1569	0.0186
SLIN	0.35694	0.48975	0.29525	0.42527
	0.1122	0.1061	0.8092	0.4005

	all lev SCP	high SCP	low SCP	medium SCP
CATE	0.18573	0.22305	0.59521	0.17193
	0.4202	0.4859	0.5941	0.7446
CATE-G	0.3601	0.3358	0.48583	0.28388
	0.1088	0.2859	0.677	0.5856
ESTIN	0.29005	0.21072	0.64894	**0.96055**
	0.2022	0.5109	0.5504	0.0023
ESLIN	**0.76311**	**0.75666**	0.73196	**-0.88498**
	0.0001	0.0044	0.4772	0.0191
G-CATE	**0.69404**	**0.85908**	-0.96977	**0.88638**
	0.0005	0.0003	0.1569	0.0186
SCP	1	1	1	1
	0	0	0	0
SLIN	0.23401	0.37094	-0.05317	0.09836
	0.3073	0.2352	0.9661	0.8529

	all lev SLIN	high SLIN	low SLIN	medium SLIN
CATE	0.18828	-0.09601	0.77079	0.73674
	0.4137	0.7666	0.4397	0.0948
CATE-G	**0.51641**	**0.70687**	0.84699	-0.00511
	0.0165	0.0102	0.3568	0.9923
ESTIN	**0.17042**	**0.24106**	**0.72526**	-0.06336
	0.4602	0.4504	0.4834	0.9051
ESLIN	-0.0819	-0.10701	0.64147	0.08842
	0.7241	0.7406	0.5567	0.8677
G-CATE	0.35694	0.48975	0.29525	0.42527
	0.1122	0.1061	0.8092	0.4005
SCP	0.23401	0.37094	-0.05317	0.09836
	0.3073	0.2352	0.9661	0.8529
SLIN	1	1	1	1
	0	0	0	0

8.5.2.2 *Linear correlation analysis calculated by AUTC for June 1999 samples group*

	all lev CAT	high CAT	low CAT	medium CAT		all lev CHIT	high CHIT	low CHIT	medium CHIT
CAT	1	1	1	1	**CAT**	-0.09106	-0.39523	0.72429	0.43621
	0	0	0	0		0.7193	0.438	0.1035	0.3872
CHIT	-0.09106	-0.39523	0.72429	0.43621	**CHIT**	1	1	1	1
	0.7193	0.438	0.1035	0.3872		0	0	0	0
GLUC	-0.2254	-0.43206	-0.7364	0.2128	**GLUC**	**0.61779**	0.79772	-0.52938	-0.21025
	0.3685	0.3922	0.0951	0.6856		0.0063	0.0572	0.2801	0.6893
PAL	**0.56341**	-0.28019	-0.09866	**0.9363**	**PAL**	0.12384	-0.31398	-0.18582	0.36077
	0.0149	0.5907	0.8525	0.006		0.6244	0.5445	0.7245	0.4823
PHEN	-0.36043	-0.22961	-0.54061	**-0.88148**	**PHEN**	-0.29341	-0.77453	-0.07467	-0.66498
	0.1418	0.6616	0.2681	0.0202		0.2373	0.0705	0.8882	0.1496
POX	0.33734	0.00792	0.71721	0.8087	**POX**	**0.79491**	**0.85122**	0.71664	0.7002
	0.171	0.9881	0.1086	0.0514		0.0001	0.0316	0.1091	0.1213
PPD-M	-0.12849	-0.21769	0.10796	-0.37257	**PPD-M**	0.01731	0.70275	0.61768	-0.36036
	0.6114	0.6786	0.8387	0.467		0.9457	0.1194	0.1913	0.4829
PPO	-0.03735	-0.08877	0.72853	**0.82928**	**PPO**	**0.8394**	**0.91022**	**0.83282**	0.13117
	0.883	0.8672	0.1005	0.0412		0.0001	0.0117	0.0396	0.8044
SCP	-0.13658	0.5575	0.15742	**-0.87207**	**SCP**	0.20552	0.43144	0.71269	-0.62814
	0.5889	0.2504	0.7658	0.0235		0.4133	0.393	0.112	0.1817
SLIN	-0.07976	0.22779	-0.25647	-0.52608	**SLIN**	**0.61789**	0.6592	0.13847	-0.07687
	0.7531	0.6642	0.6237	0.2837		0.0063	0.1544	0.7936	0.8849
TANNIN	-0.09642	-0.1413	0.20163	0.11235	**TANNIN**	-0.20376	**-0.82974**	0.43961	**-0.81875**
	0.7035	0.7895	0.7017	0.8322		0.4174	0.041	0.3831	0.0463

	all lev GLUC	high GLUC	low GLUC	medium GLUC		all lev PAL	high PAL	low PAL	medium PAL
CAT	-0.2254	-0.43206	-0.7364	0.2128	**CAT**	**0.56341**	-0.28019	-0.09866	**0.9363**
	0.3685	0.3922	0.0951	0.6856		0.0149	0.5907	0.8525	0.006
CHIT	**0.61779**	0.79772	-0.52938	-0.21025	**CHIT**	0.12384	-0.31398	-0.18582	0.36077
	0.0063	0.0572	0.2801	0.6893		0.6244	0.5445	0.7245	0.4823
GLUC	1	1	1	1	**GLUC**	0.18411	-0.15246	0.19339	0.33206
	0	0	0	0		0.4646	0.7731	0.7135	0.5202
PAL	0.18411	-0.15246	0.19339	0.33206	**PAL**	1	1	1	1
	0.4646	0.7731	0.7135	0.5202		0	0	0	0
PHEN	0.09054	-0.61556	0.45515	-0.09537	**PHEN**	-0.27646	0.35254	0.50447	**-0.89313**
	0.7209	0.1933	0.3644	0.8574		0.2668	0.4931	0.3075	0.0165
POX	**0.50139**	0.8051	-0.63737	0.17196	**POX**	0.36943	-0.53162	0.37468	**0.87344**
	0.034	0.0533	0.1734	0.7446		0.1313	0.2777	0.4643	0.023
PPD-M	-0.21321	0.56726	-0.43417	-0.75828	**PPD-M**	-0.35008	-0.47138	-0.23201	-0.5595
	0.3956	0.2404	0.3897	0.0806		0.1544	0.3453	0.6582	0.2483
PPO	**0.70963**	**0.84469**	-0.73296	0.4226	**PPO**	0.02739	-0.35674	0.19584	**0.94211**
	0.001	0.0343	0.0974	0.4038		0.9141	0.4876	0.71	0.0049
SCP	0.40547	0.43737	0.05951	0.00937	**SCP**	**-0.47654**	-0.54985	-0.05433	**-0.86172**
	0.095	0.3858	0.9108	0.986		0.0456	0.2583	0.9186	0.0274
SLIN	**0.67332**	0.52782	0.48922	-0.00806	**SLIN**	-0.20778	**-0.82294**	-0.57145	-0.66166
	0.0022	0.2818	0.3247	0.9879		0.408	0.0443	0.2361	0.1523
TANNIN	0.13331	-0.71285	0.24988	0.29824	**TANNIN**	0.15597	0.49131	-0.6201	0.22402
	0.598	0.1118	0.633	0.5659		0.5366	0.3223	0.1891	0.6696

8.5.2.2 Linear correlation analysis calculated by AUTC for June 1999 samples group

	all lev PHEN	high PHEN	low PHEN	medium PHEN		all lev POX	high POX	low POX	medium POX
CAT	-0.36043	-0.22961	-0.54061	**-0.88148**	CAT	0.33734	0.00792	0.71721	0.8087
	0.1418	0.6616	0.2681	0.0202		0.171	0.9881	0.1086	0.0514
CHIT	-0.29341	-0.77453	-0.07467	-0.66498	CHIT	**0.79491**	**0.85122**	0.71664	0.7002
	0.2373	0.0705	0.8882	0.1496		0.0001	0.0316	0.1091	0.1213
GLUC	0.09054	-0.61556	0.45515	-0.09537	GLUC	**0.50139**	0.8051	-0.63737	0.17196
	0.7209	0.1933	0.3644	0.8574		0.034	0.0533	0.1734	0.7446
PAL	-0.27646	0.35254	0.50447	-0.89313	PAL	0.36943	-0.53162	0.37468	**0.87344**
	0.2668	0.4931	0.3075	0.0165		0.1313	0.2777	0.4643	0.023
PHEN	1	1	1	1	PHEN	**-0.5148**	**-0.91216**	-0.11326	**-0.97925**
	0	0	0	0		0.0288	0.0112	0.8308	0.0006
POX	**-0.5148**	**-0.91216**	-0.11326	**-0.97925**	POX	1	1	1	1
	0.0288	0.0112	0.8308	0.0006		0	0	0	0
PPD-M	0.27791	-0.45395	0.40049	0.51544	PPD-M	-0.15175	0.55996	0.30158	-0.64878
	0.2642	0.3658	0.4314	0.2953		0.5478	0.2479	0.5613	0.1634
PPO	-0.29092	**-0.93185**	-0.06115	**-0.81359**	PPO	**0.83084**	**0.97477**	**0.95936**	0.79194
	0.2415	0.0068	0.9084	0.0489		0.0001	0.0009	0.0024	0.0604
SCP	0.39316	**-0.85321**	0.18855	**0.98868**	SCP	0.13914	**0.81918**	0.4671	**-0.94398**
	0.1065	0.0307	0.7205	0.0002		0.5819	0.0461	0.3503	0.0046
SLIN	0.18982	-0.78836	-0.10694	0.69695	SLIN	0.44195	**0.89983**	-0.35082	-0.66236
	0.4506	0.0624	0.8402	0.1238		0.0663	0.0145	0.4954	0.1518
TANNIN	**0.74646**	**0.9748**	-0.23868	0.13494	TANNIN	-0.31417	**-0.9768**	-0.17171	-0.19743
	0.0004	0.0009	0.6488	0.7988		0.2042	0.0008	0.745	0.7077

	all lev PPD-M	high PPD-M	low PPD-M	medium PPD-M		all lev PPO	high PPO	low PPO	medium PPO
CAT	-0.12849	-0.21769	0.10796	-0.37257	CAT	-0.03735	-0.08877	0.72853	**0.82928**
	0.6114	0.6786	0.8387	0.467		0.883	0.8672	0.1005	0.0412
CHIT	0.01731	0.70275	0.61768	-0.36036	CHIT	**0.8394**	**0.91022**	**0.83282**	0.13117
	0.9457	0.1194	0.1913	0.4829		0.0001	0.0117	0.0396	0.8044
GLUC	-0.21321	0.56726	-0.43417	-0.75828	GLUC	**0.70963**	**0.84469**	-0.73296	0.4226
	0.3956	0.2404	0.3897	0.0806		0.001	0.0343	0.0974	0.4038
PAL	-0.35008	-0.47138	-0.23201	-0.5595	PAL	0.02739	-0.35674	0.19584	**0.94211**
	0.1544	0.3453	0.6582	0.2483		0.9141	0.4876	0.71	0.0049
PHEN	0.27791	-0.45395	0.40049	0.51544	PHEN	-0.29092	**-0.93185**	-0.06115	**-0.81359**
	0.2642	0.3658	0.4314	0.2953		0.2415	0.0068	0.9084	0.0489
POX	-0.15175	0.55996	0.30158	-0.64878	POX	**0.83084**	**0.97477**	**0.95936**	0.79194
	0.5478	0.2479	0.5613	0.1634		0.0001	0.0009	0.0024	0.0604
PPD-M	1	1	1	1	PPD-M	0.03441	0.58084	0.53947	-0.55638
	0	0	0	0		0.8922	0.2267	0.2693	0.2515
PPO	0.03441	0.58084	0.53947	-0.55638	PPO	1	1	1	1
	0.8922	0.2267	0.2693	0.2515		0	0	0	0
SCP	0.33781	0.22303	0.44241	0.39464	SCP	0.43508	0.73684	0.50563	-0.79844
	0.1704	0.671	0.3797	0.4388		0.0712	0.0948	0.3062	0.0568
SLIN	0.16259	0.4758	-0.00878	0.25319	SLIN	**0.65766**	0.79092	-0.30946	**-0.81292**
	0.5192	0.3402	0.9868	0.6283		0.003	0.061	0.5506	0.0492
TANNIN	-0.07094	-0.55794	0.02641	0.12847	TANNIN	-0.22641	**-0.96858**	-0.09875	0.43156
	0.7797	0.2499	0.9604	0.8084		0.3663	0.0015	0.8524	0.3928

8.5.2.2 Linear correlation analysis calculated by AUTC for June 1999 samples group

	all lev SCP	high SCP	low SCP	medium SCP
CAT	-0.13658	0.5575	0.15742	**-0.87207**
	0.5889	0.2504	0.7658	0.0235
CHIT	0.20552	0.43144	0.71269	-0.62814
	0.4133	0.393	0.112	0.1817
GLUC	0.40547	0.43737	0.05951	0.00937
	0.095	0.3858	0.9108	0.986
PAL	**-0.47654**	-0.54985	-0.05433	**-0.86172**
	0.0456	0.2583	0.9186	0.0274
PHEN	0.39316	**-0.85321**	0.18855	**0.98868**
	0.1065	0.0307	0.7205	0.0002
POX	0.13914	**0.81918**	0.4671	**-0.94398**
	0.5819	0.0461	0.3503	0.0046
PPD-M	0.33781	0.22303	0.44241	0.39464
	0.1704	0.671	0.3797	0.4388
PPO	0.43508	0.73684	0.50563	-0.79844
	0.0712	0.0948	0.3062	0.0568
SCP	1	1	1	1
	0	0	0	0
SLIN	**0.73317**	**0.85269**	0.58461	0.74557
	0.0005	0.031	0.223	0.0889
TANNIN	-0.06568	**-0.85749**	0.5273	0.09788
	0.7957	0.029	0.2824	0.8536

	all lev SLIN	high SLIN	low SLIN	medium SLIN
CAT	-0.07976	0.22779	-0.25647	-0.52608
	0.7531	0.6642	0.6237	0.2837
CHIT	**0.61789**	0.6592	0.13847	-0.07687
	0.0063	0.1544	0.7936	0.8849
GLUC	**0.67332**	0.52782	0.48922	-0.00806
	0.0022	0.2818	0.3247	0.9879
PAL	-0.20778	**-0.82294**	-0.57145	-0.66166
	0.408	0.0443	0.2361	0.1523
PHEN	0.18982	-0.78836	-0.10694	0.69695
	0.4506	0.0624	0.8402	0.1238
POX	0.44195	0.89983	-0.35082	-0.66236
	0.0663	0.0145	0.4954	0.1518
PPD-M	0.16259	0.4758	-0.00878	0.25319
	0.5192	0.3402	0.9868	0.6283
PPO	**0.65766**	0.79092	-0.30946	**-0.81292**
	0.003	0.061	0.5506	0.0492
SCP	**0.73317**	**0.85269**	0.58461	0.74557
	0.0005	0.031	0.223	0.0889
SLIN	1	1	1	1
	0	0	0	0
TANNIN	0.06226	**-0.87714**	**0.83206**	-0.38254
	0.8061	0.0217	0.0399	0.4542

	all lev TANNIN	high TANNIN	low TANNIN	medium TANNIN
CAT	-0.09642	-0.1413	0.20163	0.11235
	0.7035	0.7895	0.7017	0.8322
CHIT	-0.20376	**-0.82974**	0.43961	**-0.81875**
	0.4174	0.041	0.3831	0.0463
GLUC	0.13331	-0.71285	0.24988	0.29824
	0.598	0.1118	0.633	0.5659
PAL	0.15597	0.49131	-0.6201	0.22402
	0.5366	0.3223	0.1891	0.6696
PHEN	**0.74646**	**0.9748**	-0.23868	0.13494
	0.0004	0.0009	0.6488	0.7988
POX	-0.31417	**-0.9768**	-0.17171	-0.19743
	0.2042	0.0008	0.745	0.7077
PPD-M	-0.07094	-0.55794	0.02641	0.12847
	0.7797	0.2499	0.9604	0.8084
PPO	-0.22641	**-0.96858**	-0.09875	0.43156
	0.3663	0.0015	0.8524	0.3928
SCP	-0.06568	**-0.85749**	0.5273	0.09788
	0.7957	0.029	0.2824	0.8536
SLIN	0.06226	**-0.87714**	**0.83206**	-0.38254
	0.8061	0.0217	0.0399	0.4542
TANNIN	1	1	1	1
	0	0	0	0

8.5.2.3 *Linear correlation analysis calculated by* AUTC *for December1999 samples group*

	all lev CAT	high CAT	low CAT	medium CAT
CAT	1 0	1 0	1 0	1 0
ESLIN	0.15877 0.402	0.35339 0.2598	0.66045 0.0528	-0.28185 0.4625
PHEN	-0.27055 0.1482	-0.22912 0.4738	0.08454 0.8288	**-0.75808** 0.0179
POX	**0.60857** 0.0004	0.30004 0.3434	**0.74766** 0.0206	**0.75708** 0.0182
PPD-M	0.29142 0.1182	0.56696 0.0546	0.36988 0.3272	0.05022 0.8979
PPO	**0.39664** 0.03	0.46695 0.1259	**0.72456** 0.0272	-0.2657 0.4896
SCP	-0.03252 0.8646	0.21537 0.5014	0.31772 0.4047	-0.34109 0.369
SLIN	0.28482 0.1271	0.47407 0.1195	0.3302 0.3855	0.09277 0.8124
SCPOX	0.12124 0.5233	**0.72902** 0.0071	0.53378 0.1388	-0.33346 0.3805

	all lev ESLIN	high ESLIN	low ESLIN	medium ESLIN
CAT	0.15877 0.402	0.35339 0.2598	0.66045 0.0528	-0.28185 0.4625
ESLIN	1 0	1 0	1 0	1 0
PHEN	-0.04514 0.8128	0.16832 0.601	0.50152 0.169	0.11248 0.7732
POX	0.21598 0.2517	**0.69044** 0.0129	0.58483 0.0981	-0.28418 0.4586
PPD-M	**0.60779** 0.0004	0.47179 0.1215	0.53577 0.1371	0.54598 0.1283
PPO	0.24146 0.1986	0.16595 0.6062	0.35276 0.3518	0.01671 0.966
SCP	**0.60733** 0.0004	**0.73703** 0.0062	0.01962 0.96	**0.78021** 0.0131
SLIN	**0.42711** 0.0186	0.26431 0.4065	**0.72365** 0.0275	0.2801 0.4654
SCPOX	**0.42745** 0.0185	**0.63277** 0.0272	**0.8945** 0.0011	-0.0393 0.92

	all lev PHEN	high PHEN	low PHEN	medium PHEN
CAT	-0.27055 0.1482	-0.22912 0.4738	0.08454 0.8288	**-0.75808** 0.0179
ESLIN	-0.04514 0.8128	0.16832 0.601	0.50152 0.169	0.11248 0.7732
PHEN	1 0	1 0	1 0	1 0
POX	-0.28293 0.1298	0.19525 0.5431	-0.24685 0.522	**-0.85007** 0.0037
PPD-M	-0.32066 0.0841	-0.05244 0.8714	-0.34549 0.3625	-0.43904 0.2371
PPO	-0.3127 0.0925	-0.47439 0.1192	-0.14592 0.708	-0.15187 0.6965
SCP	-0.2019 0.2846	0.12779 0.6923	-0.47688 0.1943	-0.10551 0.787
SLIN	-0.03475 0.8554	**-0.63435** 0.0267	**0.92097** 0.0004	0.25445 0.5088
SCPOX	0.13474 0.4778	-0.0446 0.8905	0.59625 0.0901	0.22665 0.5576

	all lev POX	high POX	low POX	medium POX
CAT	**0.60857** 0.0004	0.30004 0.3434	**0.74766** 0.0206	**0.75708** 0.0182
ESLIN	0.21598 0.2517	**0.69044** 0.0129	0.58483 0.0981	-0.28418 0.4586
PHEN	-0.28293 0.1298	0.19525 0.5431	-0.24685 0.522	**-0.85007** 0.0037
POX	1 0	1 0	1 0	1 0
PPD-M	0.34034 0.0657	0.54075 0.0695	**0.77219** 0.0147	0.38952 0.3001
PPO	**0.50993** 0.004	-0.02654 0.9347	**0.80251** 0.0092	-0.0751 0.8477
SCP	0.10231 0.5906	0.56089 0.0578	0.12735 0.744	0.0362 0.9263
SLIN	0.05259 0.7825	0.35242 0.2612	-0.03692 0.9249	-0.17403 0.6543
SCPOX	0.19996 0.2894	**0.84357** 0.0006	0.54335 0.1306	-0.12686 0.745

8.5.1.3 Linear correlation analysis calculated by AUTC for December1999 samples group

	all lev PPD-M	high PPD-M	low PPD-M	medium PPD-M		all lev PPO	high PPO	low PPO	medium PPO
CAT	0.29142	0.56696	0.36988	0.05022	**CAT**	**0.39664**	0.46695	**0.72456**	-0.2657
	0.1182	0.0546	0.3272	0.8979		0.03	0.1259	0.0272	0.4896
ESLIN	**0.60779**	0.47179	0.53577	0.54598	**ESLIN**	0.24146	0.16595	0.35276	0.01671
	0.0004	0.1215	0.1371	0.1283		0.1986	0.6062	0.3518	0.966
PHEN	-0.32066	-0.05244	-0.34549	-0.43904	**PHEN**	-0.3127	-0.47439	-0.14592	-0.15187
	0.0841	0.8714	0.3625	0.2371		0.0925	0.1192	0.708	0.6965
POX	0.34034	0.54075	**0.77219**	0.38952	**POX**	**0.50993**	-0.02654	**0.80251**	-0.0751
	0.0657	0.0695	0.0147	0.3001		0.004	0.9347	0.0092	0.8477
PPD-M	1	1	1	1	**PPD-M**	0.20399	0.05514	0.39901	-0.04926
	0	0	0	0		0.2796	0.8649	0.2874	0.8999
PPO	0.20399	0.05514	0.39901	-0.04926	**PPO**	1	1	1	1
	0.2796	0.8649	0.2874	0.8999		0	0	0	0
SCP	**0.58541**	0.5515	0.1352	**0.75059**	**SCP**	0.07314	-0.01102	0.12795	0.20949
	0.0007	0.0631	0.7287	0.0198		0.7009	0.9729	0.7429	0.5885
SLIN	**0.44596**	0.45769	-0.0747	-0.3872	**SLIN**	**0.38441**	**0.71663**	-0.01107	0.00191
	0.0135	0.1346	0.8485	0.3032		0.036	0.0087	0.9775	0.9961
SCPOX	**0.52785**	**0.6413**	0.34518	0.33885	**SCPOX**	0.1506	0.35647	0.41727	-0.00016
	0.0027	0.0246	0.3629	0.3724		0.427	0.2554	0.2638	0.9997

	all lev SCP	high SCP	low SCP	medium SCP		all lev SLIN	high SLIN	low SLIN	medium SLIN
CAT	-0.03252	0.21537	0.31772	-0.34109	**CAT**	0.28482	0.47407	0.3302	0.09277
	0.8646	0.5014	0.4047	0.369		0.1271	0.1195	0.3855	0.8124
ESLIN	**0.60733**	**0.73703**	0.01962	**0.78021**	**ESLIN**	**0.42711**	0.26431	**0.72365**	0.2801
	0.0004	0.0062	0.96	0.0131		0.0186	0.4065	0.0275	0.4654
PHEN	-0.2019	0.12779	-0.47688	-0.10551	**PHEN**	-0.03475	**-0.63435**	**0.92097**	0.25445
	0.2846	0.6923	0.1943	0.787		0.8554	0.0267	0.0004	0.5088
POX	0.10231	0.56089	0.12735	0.0362	**POX**	0.05259	0.35242	-0.03692	-0.17403
	0.5906	0.0578	0.744	0.9263		0.7825	0.2612	0.9249	0.6543
PPD-M	**0.58541**	0.5515	0.1352	**0.75059**	**PPD-M**	0.44596	0.45769	-0.0747	-0.3872
	0.0007	0.0631	0.7287	0.0198		0.0135	0.1346	0.8485	0.3032
PPO	0.07314	-0.01102	0.12795	0.20949	**PPO**	**0.38441**	**0.71663**	-0.01107	0.00191
	0.7009	0.9729	0.7429	0.5885		0.036	0.0087	0.9775	0.9961
SCP	1	1	1	1	**SCP**	0.12588	0.10636	-0.26452	-0.16427
	0	0	0	0		0.5074	0.7422	0.4916	0.6728
SLIN	0.12588	0.10636	-0.26452	-0.16427	**SLIN**	1	1	1	1
	0.5074	0.7422	0.4916	0.6728		0	0	0	0
SCPOX	0.20502	0.48803	-0.22121	-0.03978	**SCPOX**	**0.42159**	**0.59332**	**0.69761**	-0.19436
	0.2771	0.1075	0.5673	0.9191		0.0203	0.042	0.0367	0.6163

8.5.1.3 Linear correlation analysis calculated by AUTC for December1999 samples group

	all lev SCPOX	high SCPOX	low SCPOX	medium SCPOX
CAT	0.12124	**0.72902**	0.53378	-0.33346
	0.5233	0.0071	0.1388	0.3805
ESLIN	**0.42745**	**0.63277**	**0.8945**	-0.0393
	0.0185	0.0272	0.0011	0.92
PHEN	0.13474	-0.0446	0.59625	0.22665
	0.4778	0.8905	0.0901	0.5576
POX	0.19996	**0.84357**	0.54335	-0.12686
	0.2894	0.0006	0.1306	0.745
PPD-M	**0.52785**	**0.6413**	0.34518	0.33885
	0.0027	0.0246	0.3629	0.3724
PPO	0.1506	0.35647	0.41727	-0.00016
	0.427	0.2554	0.2638	0.9997
SCP	0.20502	0.48803	-0.22121	-0.03978
	0.2771	0.1075	0.5673	0.9191
SLIN	**0.42159**	**0.59332**	**0.69761**	-0.19436
	0.0203	0.042	0.0367	0.6163
SCPOX	1	1	1	1
	0	0	0	0

8.5.2.4 *Linear correlation analysis calculated by* AUTC *for Family K samples group*

	all lev v	low ESLIN	medium ESLIN	high ESLIN		all lev PHEN	low PHEN	medium PHEN	high PHEN
ESLIN	1	1	1	1	**ESLIN**	-0.09947	-0.24131	0.05582	0.00499
	0	0	0	0		0.3127	0.1989	0.7696	0.974
PHEN	-0.09947	-0.24131	0.05582	0.00499	**PHEN**	1	1	1	1
	0.3127	0.1989	0.7696	0.974		0	0	0	0
POX	0.05586	**0.49899**	0.00621	0.07506	**POX**	-0.10672	-0.22839	-0.0928	-0.15371
	0.5714	0.005	0.974	0.6241		0.2786	0.2248	0.6257	0.3134
PPD-M	**0.74474**	**0.72589**	**0.77006**	**0.56688**	**PPD-M**	-0.08346	-0.03446	0.03072	-0.00453
	0.0001	0.0001	0.0001	0.0001		0.3973	0.8565	0.872	0.9764
PPO	0.0481	-0.10869	-0.05399	0.3466	**PPO**	-0.00599	0.02953	-0.11145	0.01615
	0.6261	0.5675	0.7769	0.0197		0.9517	0.8769	0.5577	0.9161
SCP	0.08516	**0.36148**	-0.11622	-0.16637	**SCP**	0.02478	0.13874	0.06314	0.11113
	0.3877	0.0497	0.5408	0.2747		0.8019	0.4647	0.7403	0.4674
SLIN	**0.2999**	**0.43036**	0.1595	0.24901	**SLIN**	-0.18719	0.07168	-0.17147	-0.28556
	0.0019	0.0176	0.3999	0.099		0.0559	0.7066	0.3649	0.0572
SCPOX	**0.53892**	**0.59949**	**0.50373**	**0.5644**	**SCPOX**	-0.09726	-0.08236	-0.07303	-0.02707
	0.0001	0.0005	0.0045	0.0001		0.3236	0.6653	0.7013	0.8599

	all lev POX	low POX	medium POX	high POX		all lev PPD-M	low PPD-M	medium PPD-M	high PPD-M
ESLIN	0.05586	**0.49899**	0.00621	0.07506	**ESLIN**	**0.74474**	**0.72589**	**0.77006**	**0.56688**
	0.5714	0.005	0.974	0.6241		0.0001	0.0001	0.0001	0.0001
PHEN	-0.10672	-0.22839	-0.0928	-0.15371	**PHEN**	-0.08346	-0.03446	0.03072	-0.00453
	0.2786	0.2248	0.6257	0.3134		0.3973	0.8565	0.872	0.9764
POX	1	1	1	1	**POX**	0.07562	0.30281	0.19503	0.10923
	0	0	0	0		0.4433	0.1038	0.3017	0.4751
PPD-M	0.07562	0.30281	0.19503	0.10923	**PPD-M**	1	1	1	1
	0.4433	0.1038	0.3017	0.4751		0	0	0	0
PPO	0.03384	-0.2164	0.3265	0.10975	**PPO**	0.11038	-0.12386	0.12271	**0.32879**
	0.7318	0.2507	0.0782	0.4729		0.2623	0.5143	0.5183	0.0274
SCP	0.06039	0.07911	-0.10808	0.00332	**SCP**	-0.05697	0.34186	-0.23058	**-0.448**
	0.5406	0.6778	0.5697	0.9828		0.5638	0.0645	0.2202	0.002
SLIN	0.03354	0.17062	**0.37879**	0.02355	**SLIN**	0.15594	0.2105	0.20857	-0.15095
	0.7341	0.3673	0.039	0.878		0.1122	0.2642	0.2687	0.3223
SCPOX	0.16125	0.34513	0.59328	0.22879	**SCPOX**	**0.52738**	**0.66463**	**0.55016**	**0.49993**
	0.1003	0.0618	0.0005	0.1306		0.0001	0.0001	0.0016	0.0005

8.5.1.4 Linear correlation analysis calculated by AUTC for Family K samples group

	all lev PPO	low PPO	medium PPO	high PPO		all lev SCP	low SCP	medium SCP	high SCP
ESLIN	0.0481	-0.10869	-0.05399	**0.3466**	**ESLIN**	0.08516	0.36148	-0.11622	-0.16637
	0.6261	0.5675	0.7769	0.0197		0.3877	0.0497	0.5408	0.2747
PHEN	-0.00599	0.02953	-0.11145	0.01615	**PHEN**	0.02478	0.13874	0.06314	0.11113
	0.9517	0.8769	0.5577	0.9161		0.8019	0.4647	0.7403	0.4674
POX	0.03384	-0.2164	0.3265	0.10975	**POX**	0.06039	0.07911	-0.10808	0.00332
	0.7318	0.2507	0.0782	0.4729		0.5406	0.6778	0.5697	0.9828
PPD-M	0.11038	-0.12386	0.12271	**0.32879**	**PPD-M**	-0.05697	0.34186	-0.23058	**-0.448**
	0.2623	0.5143	0.5183	0.0274		0.5638	0.0645	0.2202	0.002
PPO	1	1	1	1	**PPO**	**-0.42923**	**-0.50623**	**-0.38004**	**-0.41551**
	0	0	0	0		0.0001	0.0043	0.0383	0.0045
SCP	**-0.42923**	**-0.50623**	**-0.38004**	**-0.41551**	**SCP**	1	1	1	1
	0.0001	0.0043	0.0383	0.0045		0	0	0	0
SLIN	0.06479	0.24437	-0.03572	0.02311	**SLIN**	**0.42509**	0.26116	**0.39962**	**0.41711**
	0.5114	0.1931	0.8514	0.8802		0.0001	0.1633	0.0287	0.0044
SCPOX	0.10875	-0.05724	0.11661	**0.39115**	**SCPOX**	0.09145	0.20356	0.08962	-0.2357
	0.2695	0.7638	0.5395	0.0079		0.3535	0.2806	0.6377	0.1191

	all lev SLIN	low SLIN	medium SLIN	high SLIN		all lev SCPOX	low SCPOX	medium SCPOX	high SCPOX
ESLIN	**0.2999**	**0.43036**	0.1595	0.24901	**ESLIN**	**0.53892**	**0.59949**	**0.50373**	**0.5644**
	0.0019	0.0176	0.3999	0.099		0.0001	0.0005	0.0045	0.0001
PHEN	-0.18719	0.07168	-0.17147	-0.28556	**PHEN**	-0.09726	-0.08236	-0.07303	-0.02707
	0.0559	0.7066	0.3649	0.0572		0.3236	0.6653	0.7013	0.8599
POX	0.03354	0.17062	**0.37879**	0.02355	**POX**	0.16125	0.34513	**0.59328**	0.22879
	0.7341	0.3673	0.039	0.878		0.1003	0.0618	0.0005	0.1306
PPD-M	0.15594	0.2105	0.20857	-0.15095	**PPD-M**	**0.52738**	**0.66463**	**0.55016**	**0.49993**
	0.1122	0.2642	0.2687	0.3223		0.0001	0.0001	0.0016	0.0005
PPO	0.06479	0.24437	-0.03572	0.02311	**PPO**	0.10875	-0.05724	0.11661	**0.39115**
	0.5114	0.1931	0.8514	0.8802		0.2695	0.7638	0.5395	0.0079
SCP	**0.42509**	0.26116	**0.39962**	**0.41711**	**SCP**	0.09145	0.20356	0.08962	-0.2357
	0.0001	0.1633	0.0287	0.0044		0.3535	0.2806	0.6377	0.1191
SLIN	1	1	1	1	**SLIN**	**0.30694**	0.22019	**0.44567**	0.14861
	0	0	0	0		0.0014	0.2423	0.0136	0.3299
SCPOX	**0.30694**	0.22019	**0.44567**	0.14861	**SCPOX**	1	1	1	1
	0.0014	0.2423	0.0136	0.3299		0	0	0	0

8.6 PRINCIPAL COMPONENT ANALYSIS

Principal component analysis is a multivariate technique for examining relationships among several quantitative variables. Given a table with p numeric columns, p principal components can be computed. Each principal component is a linear combination of the original columns, with coefficients equal to the eigenvectors of the correlation or covariance matrix. The eigenvectors are customarily taken with unit length. The principal components are sorted by descending order of the eigenvalues, which are equal to the variances of the components.

A Principal component analysis (PCA) was used to determine the main biochemical trait in the PPD response. The PCA was performed only for the December 1999 sample group. PCA was performed using the PRINCOMP procedure of SAS. In the following tables, five and four principal components are reported for secondary metabolites and enzymes, respectively. Values for each variable in all components are given

8.6.1 Principal component analysis for secondary metabolites in December 1999 samples group

DAY 0

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	-0.1855	0	-0.1277	-0.1202
PHEN	-0.1855	1	0	0.2746	0.6424
PPD-M	0	0	0	0	0
SCP	-0.1277	0.2746	0	1	0.3505
SLIN	-0.1202	0.6424	0	0.3505	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	1.93479	0.996752	0.48369	0.4837
PRIN2	0.93804	0.158372	0.23451	0.71821
PRIN3	0.77967	0.43217	0.19491	0.91313
PRIN4	0.3475	0.347499	0.08687	1
PRIN5	0		0	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	-0.258603	0.960605	0.057345	0.084111	0
PHEN	0.602099	0.12601	-0.395774	0.681881	0
PPD-M	0	0	0	0	1
SCP	0.441872	0.055891	0.887936	0.11487	0
SLIN	0.612658	0.241321	-0.227256	-0.717474	0

DAY 1

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	0.2395	0.7664	0.3888	0.0795
PHEN	0.2395	1	-0.0909	-0.1887	-0.027
PPD-M	0.7664	-0.0909	1	0.7571	0.1677
SCP	0.3888	-0.1887	0.7571	1	0.1553
SLIN	0.0795	-0.027	0.1677	0.1553	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.33164	1.15188	0.46632	0.46633
PRIN2	1.17976	0.22783	0.23595	0.70228
PRIN3	0.95193	0.50188	0.19038	0.89267
PRIN4	0.45005	0.36343	0.09001	0.98268
PRIN5	0.08662		0.01732	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	0.527039	0.402701	-0.06471	-0.547358	0.506235
PHEN	-0.028273	0.853555	0.213105	0.456921	-0.128275
PPD-M	0.633338	-0.017047	-0.103796	-0.094166	-0.760889
SCP	0.538344	-0.265881	-0.115559	0.691752	0.38421
SLIN	0.17464	-0.195684	0.96243	-0.065072	0.026512

DAY 2

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	-0.3462	0.8817	-0.2692	0.5346
PHEN	-0.3462	1	-0.2535	-0.0877	0.0771
PPD-M	0.8817	-0.2535	1	-0.2061	0.3131
SCP	-0.2692	-0.0877	-0.2061	1	-0.3554
SLIN	0.5346	0.0771	0.3131	-0.3554	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.41011	1.15432	0.48202	0.48202
PRIN2	1.25579	0.55534	0.25115	0.73318
PRIN3	0.70045	0.13526	0.14008	0.87327
PRIN4	0.56519	0.49671	0.11303	0.98631
PRIN5	0.06847		0.01369	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	0.616759	-0.135867	0.154989	0.086195	0.754783
PHEN	-0.210834	0.688127	0.512381	0.447137	0.139871
PPD-M	0.559557	-0.193181	0.213362	0.502198	-0.59317
SCP	-0.292339	-0.534651	0.767911	-0.19734	0.00749
SLIN	0.420227	0.429943	0.279702	-0.708162	-0.242553

DAY 3

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	-0.0774	0.8767	0.4173	0.4886
PHEN	-0.0774	1	-0.1725	-0.126	-0.1951
PPD-M	0.8767	-0.1725	1	0.4425	0.5722
SCP	0.4173	-0.126	0.4425	1	0.3748
SLIN	0.4886	-0.1951	0.5722	0.3748	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.66946	1.69409	0.53389	0.53389
PRIN2	0.97537	0.28849	0.19507	0.72897
PRIN3	0.68688	0.13215	0.13737	0.86634
PRIN4	0.55473	0.44116	0.11094	0.97729
PRIN5	0.11357		0.02271	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	0.536472	0.237675	-0.284835	-0.35515	-0.669661
PHEN	-0.16657	0.959653	0.088637	0.198323	0.064277
PPD-M	0.561681	0.122255	-0.272701	-0.235999	0.734511
SCP	0.398481	0.008824	0.910103	-0.113253	-0.004683
SLIN	0.458453	-0.086904	-0.091432	0.875223	-0.088851

DAY 4

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	0.2429	0.8167	0.7482	0.0502
PHEN	0.2429	1	0.0091	0.3952	-0.1434
PPD-M	0.8167	0.0091	1	0.6992	0.2409
SCP	0.7482	0.3952	0.6992	1	0.057
SLIN	0.0502	-0.1434	0.2409	0.057	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	PRIN1	2.61429	1.38892	0.52285	0.52286
PRIN2	PRIN2	1.22537	0.43808	0.24507	0.76793
PRIN3	PRIN3	0.7873	0.5486	0.15745	0.92539
PRIN4	PRIN4	0.2387	0.10436	0.04774	0.97313
PRIN5	PRIN5	0.13434		0.02686	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	0.572918	-0.00239	-0.195931	0.504691	-0.615352
PHEN	0.217474	-0.661916	0.635254	0.256265	0.21296
PPD-M	0.54856	0.277542	-0.22027	0.187659	0.733701
SCP	0.559933	-0.134552	0.042107	-0.802591	-0.149822
SLIN	0.100136	0.683174	0.712581	0.015747	-0.123395

CURVE AREA

Correlation Matrix

	ESLIN	PHEN	PPD-M	SCP	SLIN
ESLIN	1	-0.0451	0.8712	0.6302	0.3769
PHEN	-0.0451	1	-0.3242	-0.2021	-0.0468
PPD-M	0.8712	-0.3242	1	0.7845	0.4078
SCP	0.6302	-0.2021	0.7845	1	-0.0391
SLIN	0.3769	-0.0468	0.4078	-0.0391	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.71582	1.64365	0.54316	0.54316
PRIN2	1.07217	0.13285	0.21443	0.7576
PRIN3	0.93932	0.70708	0.18786	0.94546
PRIN4	0.23224	0.19179	0.04644	0.99191
PRIN5	0.04045		0.00809	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4	PRIN5
ESLIN	0.545671	0.178173	0.220355	-0.671744	-0.413161
PHEN	-0.192307	0.508026	0.810509	0.141553	0.167229
PPD-M	0.598781	-0.00965	-0.014725	-0.013408	0.800607
SCP	0.490998	-0.379787	0.31935	0.621534	-0.355517
SLIN	0.256211	0.752219	-0.43854	0.377145	-0.184305

8.6.2 Principal component analysis for enzymatic activity in December 1999 samples group

DAY 0

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.6575	-0.0404	-0.3355
POX	0.6575	1	0.0008	-0.1232
PPO	-0.0404	0.0008	1	0.5766
SCP_POX	-0.3355	-0.1232	0.5766	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	1.89239	0.502232	0.473097	0.4731
PRIN2	1.39016	0.938578	0.347539	0.82064
PRIN3	0.45158	0.185698	0.112894	0.93353
PRIN4	0.26588	.	0.06647	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.583383	0.378988	-0.320546	0.642871
POX	0.501857	0.496246	0.506305	-0.495515
PPO	-0.364677	0.634652	-0.59151	-0.338147
SCP_POX	-0.524227	0.45533	0.539464	0.476276

DAY 1

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.5141	0.3693	0.1021
POX	0.5141	1	0.5964	-0.1694
PPO	0.3693	0.5964	1	-0.2503
SCP_POX	0.1021	-0.1694	-0.2503	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.03047	0.933318	0.507617	0.50762
PRIN2	1.09715	0.593193	0.274288	0.78191
PRIN3	0.50396	0.135539	0.12599	0.9079
PRIN4	0.36842	.	0.092105	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.496196	0.480353	-0.632475	0.35075
POX	0.61556	0.037212	0.076703	-0.783466
PPO	0.580999	-0.163427	0.613868	0.508821
SCP_POX	-0.193173	0.860911	0.46611	-0.06525

DAY 2

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.5008	0.5995	-0.1626
POX	0.5008	1	0.7273	-0.0212
PPO	0.5995	0.7273	1	0.0876
SCP_POX	-0.1626	-0.0212	0.0876	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.22495	1.17382	0.556237	0.55624
PRIN2	1.05113	0.56656	0.262783	0.81902
PRIN3	0.48457	0.24523	0.121144	0.94016
PRIN4	0.23934	.	0.059836	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.540011	-0.213594	0.767869	0.270448
POX	0.581809	0.073475	-0.585764	0.559446
PPO	0.606971	0.180123	-0.106737	-0.766648
SCP_POX	-0.038371	0.957358	0.236356	0.161644

DAY 3

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.6295	0.4965	0.2224
POX	0.6295	1	0.47	0.3119
PPO	0.4965	0.47	1	0.4068
SCP_POX	0.2224	0.3119	0.4068	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.2928	1.45172	0.573201	0.5732
PRIN2	0.84109	0.32883	0.210272	0.78347
PRIN3	0.51226	0.15841	0.128065	0.91154
PRIN4	0.35385	.	0.088462	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.531558	-0.446739	0.081479	0.715005
POX	0.542352	-0.297115	0.455063	-0.640699
PPO	0.523079	0.147464	-0.814278	-0.203946
SCP_POX	0.386896	0.830904	0.351042	0.191519

DAY 4

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.6665	0.409	0.0116
POX	0.6665	1	0.4876	0.2852
PPO	0.409	0.4876	1	0.3922
SCP_POX	0.0116	0.2852	0.3922	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.18177	1.1425	0.545444	0.54544
PRIN2	1.03928	0.53725	0.259819	0.80526
PRIN3	0.50203	0.22511	0.125507	0.93077
PRIN4	0.27692	.	0.06923	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.518453	-0.522121	0.120006	0.66648
POX	0.588762	-0.186466	0.401417	-0.676353
PPO	0.529466	0.2311	-0.811846	-0.084647
SCP_POX	0.322848	0.799508	0.406657	0.30197

CURVE AREA

Correlation Matrix

	CAT	POX	PPO	SCP_POX
CAT	1	0.6902	0.5342	-0.0587
POX	0.6902	1	0.616	-0.0657
PPO	0.5342	0.616	1	0.2485
SCP_POX	-0.0587	-0.0657	0.2485	1

Eigenvalues of the Correlation Matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN1	2.23251	1.13332	0.558129	0.55813
PRIN2	1.09919	0.71176	0.274798	0.83293
PRIN3	0.38743	0.10656	0.096857	0.92978
PRIN4	0.28087	.	0.070217	1

Eigenvectors

	PRIN1	PRIN2	PRIN3	PRIN4
CAT	0.573723	-0.187084	0.671789	-0.429583
POX	0.597275	-0.166096	-0.055862	0.782659
PPO	0.557908	0.281663	-0.662288	-0.413255
SCP_POX	0.053326	0.926325	0.327039	0.179232

8.7 EVALUATION OF PPD RESPONSE IN FAMILY K POPULATION

An analysis of variance (ANOVA) was performed to study the PPD scores of Family K cultivars of in two different localities (Quilichao and Palmira) during 1998 and 1999. Sources of variation were: replicates (REP), genotypes (GEN), localities (SITE), year (YEAR) and the interactions GEN*SITE, and GEN*YEAR.
Means of PPD scores were separated by the REGWQ test.

8.7.1 ANOVA of PPD scores of Family K in two different localities during 1998

SITE=PALMIRA YEAR=98					
Class	Levels	Values			
REP	3	1 2 3			
GENOT	144				
Source	DF	SS	MS	F	Pr > F
Model	425	138.3295445	0.32548128	3.45	0.0001
REP	2	4.35532658	2.17766329	10.28	0.0001
GENOT	142	71.91659829	0.50645492	2.39	0.0001
REP*GENOT	281	59.52667516	0.2118387	2.25	0.0001
Error	1939	182.9518531	0.09435371		
Total	2364	321.2813976			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.430556	54.22704	0.3071705	0.5664526

SITE=QUILICHAO YEAR=98					
Class	Levels	Values			
REP	3	1 2 3			
GENOT	144				
Source	DF	SS	MS	F	Pr > F
Model	427	94.52461821	0.22136913	5.15	0.0001
REP	2	8.96194972	4.48097486	39.39	0.0001
GENOT	143	52.49911037	0.36712665	3.23	0.0001
REP*GENOT	282	32.08240199	0.11376738	2.65	0.0001
Error	2061	88.54579674	0.04296254		
Total	2488	183.070415			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.516329	99.91071	0.2072741	0.2074593

SITE=PALMIRA&QUILICHAO YEAR=98					
Class	Levels	Values			
SITE	2	PAL QUIL			
REP	3	1 2 3			
GENOT	144				
Source	DF	SS	MS	F	Pr > F
Model	853	389.1433571	0.45620558	6.72	0.0001
SITE	1	136.1701523	136.1701523	40.9	0.0031
REP(SITE)	4	13.3172763	3.32931908	49.05	0.0001
GENOT	143	90.81223306	0.63505058	9.36	0.0001
SITE*GENOT	142	34.2100172	0.24091561	3.55	0.0001
REP*GENOT(SITE)	563	91.60907714	0.16271595	2.4	0.0001
Error	4000	271.4976498	0.06787441		
Total	4853	660.6410069			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.589039	68.13474	0.2605272	0.3823706

8.7.2 ANOVA of PPD scores of Family K in two different localities during 1999

SITE=PALMIRA YEAR=99					
Class	Levels	Values			
REP	3	1 2 3			
GENOT	147				
Source	DF	SS	MS	F	Pr > F
Model	387	190.7404215	0.49286931	11.47	0.0001
REP	2	3.83921079	1.9196054	7.59	0.0006
GENOT	140	113.0520835	0.80751488	3.19	0.0001
REP*GENOT	245	61.95161866	0.25286375	5.89	0.0001
Error	3180	136.5863811	0.04295169		
Total	3567	327.3268025			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.582722	47.22668	0.2072479	0.4388365

SITE=QUILICHAO YEAR=99					
Class	Levels	Values			
REP	3	1 2 3			
GENOT	147				
Source	DF	SS	MS	F	Pr > F
Model	418	151.4884303	0.36241251	15.11	0.0001
REP	2	4.98490821	2.49245411	20.63	0.0001
GENOT	141	111.2262118	0.78883838	6.53	0.0001
REP*GENOT	275	33.22636425	0.12082314	5.04	0.0001
Error	4278	102.6028269	0.02398383		
Total	4696	254.0912572			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.596197	62.36891	0.1548671	0.2483082

SITE=PALMIRA&QUILICHAO YEAR=99					
Class	Levels	Values			
SITE	2	PAL QUIL			
REP	3	1 2 3			
GENOT	147				
Source	DF	SS	MS	F	Pr > F
Model	806	415.8362641	0.51592589	16.09	0.0001
SITE	1	42.73477607	42.73477607	19.37	0.0117
REP(SITE)	4	8.824119	2.20602975	68.78	0.0001
GENOT	143	159.5322781	1.11561034	34.79	0.0001
SITE*GENOT	138	63.23078653	0.45819411	14.29	0.0001
REP*GENOT(SITE)	520	95.17798291	0.18303458	5.71	0.0001
Error	7458	239.1892079	0.03207149		
Total	8264	655.025472			
		R-MS	C.V.	R-MSE	TPCT Mean
		0.63484	54.17641	0.1790852	0.3305593

8.7.3 ANOVA of PPD scores of Family K in two different localities during 1999 and 1999

SITE=PALMIRA YEAR=98&99					
Class	Levels	Values			
YEAR	2	98 99			
REP	3	1 2 3			
GENOT	147				
Source	DF	SS	MS	F	Pr > F
Model	813	352.2328454	0.43325073	6.94	0.0001
YEAR	1	25.74613778	25.74613778	12.57	0.0239
REP(YEAR)	4	8.19453737	2.04863434	32.82	0.0001
GENOT	145	112.9437177	0.77892219	12.48	0.0001
YEAR*GENOT	137	51.27094432	0.37424047	6	0.0001
REP*GENOT(YEAR)	526	121.4782938	0.23094733	3.7	0.0001
Error	5119	319.5382341	0.062422		
Total	5932	671.7710796			
		R-Square	C.V.	R-MSE	TPCT Mean
		0.524335	51.01911	0.249844	0.4897066

SITE=QUILICHAO YEAR=98&99					
Class	Levels	Values			
YEAR	2	98 99			
REP	3	1 2 3			
GENOT	147				
Source	DF	SS	MS	F	Pr > F
Model	846	248.7277305	0.29400441	9.75	0.0001
YEAR	1	1.45792512	1.45792512	0.42	0.5531
REP(YEAR)	4	13.94685793	3.48671448	115.63	0.0001
GENOT	146	115.2197419	0.78917631	26.17	0.0001
YEAR*GENOT	138	28.55689755	0.20693404	6.86	0.0001
REP*GENOT(YEAR)	557	65.30876623	0.11725093	3.89	0.0001
Error	6339	191.1486236	0.03015438		
Total	7185	439.8763541			
		R-Square	C.V.	R-MSE	TPCT Mean
		0.565449	74.15893	0.1736502	0.2341595

8.7.4 REGWQ grouping by PPD scores of Family K in two different localities during 1998 and 1999

YEAR=99		
GROUP	MEAN	SITE
A	0.43884	PAL
B	0.24831	QUIL

SITE=PALMIRA		
GROUP	MEAN	YEAR
A	0.56645	98
B	0.43884	99

YEAR=98		
GROUP	MEAN	SITE
A	0.56645	PAL
B	0.20746	QUIL

SITE=QUILICHAO		
GROUP	MEAN	YEAR
A	0.24831	99
A	0.20746	98